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Regulation of Crbp1 In Mammary Epithelial Cells

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REGULATION OF CRBP1 IN MAMMARY EPITHELIAL CELLS

A Thesis Presented

By

STACY L. PEASE

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Molecular and Cellular Biology

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Completing this thesis has been a long time coming for me. During the process, there were several times when I contemplated leaving it behind, but in those moments I would remember all of the people who had a part in making this project happen and they motivated me to continue. Although I have left the world of science to pursue a career in business, I carry with me many rich experiences and lessons from my years in the lab.

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ABSTRACT

REGULATION OF CRBP1 IN MAMMARY EPITHELIAL CELLS

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Breast cancer is the second leading cause of death of women in the United States, warranting further investigation into preventative therapies. It has been well documented that early pregnancy results in a lifetime decreased risk of breast cancer in humans and mounting evidence suggests that the retinoic acid pathway may play an important role in this protective effect. Cellular retinol binding protein-1 (CRBP1) is an essential component of the retinoic acid pathway and we propose that it plays an important role in pregnancy-induced protection against breast cancer. In order to investigate the role of CRBP1 in parity-induced protection against breast cancer, we utilized both mouse and human mammary epithelial cells. We examined the effect that pregnancy has on CRBP1 expression, how CRBP1 is regulated by growth promoting and inhibiting agents, if loss of CRBP1 is essential for the induction of the apoptotic pathway, and how CpG methylation of

key breast cancer genes relates to known risk factors for the disease. Based on our study, CRBP1 is persistently upregulated in response to pregnancy in the mouse mammary gland at both the RNA and protein levels. Using a cell culture model, we established that CRBP1 is regulated by chemical agents that both promote and inhibit cellular growth. Utilizing CRBP1 knockout mice, we demonstrated that CRBP1 is not essential for induction of radiation induced apoptosis in parous mice. Finally, through methylation analysis, we examined how known breast cancer risk factors correlate to CpG methylation of three important genes for breast cancer and noted interesting trends that warrant future study.

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CHAPTER 1

OVERALL INTRODUCTION

Breast cancer is the second leading cause of cancer related death in women in the United States, resulting in an excess of 40,000 deaths in 2008 (Ries et al. 2008). Although advances in breast cancer therapies have improved life expectancy for non-metastatic breast cancer, cure rates for metastasized breast cancer remain low. In light of this, it is imperative that the development of preventative therapies against breast cancer be a research focus. One approach to breast cancer prevention is through a better understanding of parity-induced protection. It has been well documented that women who have a full term pregnancy before the age of twenty-four have a decreased lifetime risk of developing breast cancer and that additional pregnancies increase this protection (Russo et al. 2005). This protective effect has also been observed in rodents. It has been shown that pregnancy results in mammary gland differentiation, as well as persistent changes to gene expression (Verlinden et al. 2005). Despite these observations, the molecular mechanisms responsible for this effect are largely unknown.

Mounting evidence suggests that the retinoic acid pathway may be important in parity-induced protection against breast cancer. The retinoic acid pathway is responsible for converting exogenous Vitamin A into retinoic acid that the body can store and later utilize. It has been shown that retinoids are important for the regulation of cell proliferation, mammary gland differentiation, and apoptosis and that components of this pathway are upregulated in response to early pregnancy (Verlinden et al. 2005). Loss of retinoic acid signaling has been implicated in breast cancer development and retinoic acid

signaling has been proven necessary for proper mammary gland morphogenesis (Wang et al. 2005). Clinically, all-trans retinoic acid (ATRA) has been used as a chemotherapeutic and chemopreventative agent in the treatment of several cancers. Collectively, this evidence suggests that regulation of the retinoic acid pathway is important in the prevention of breast cancer.

Cellular retinol-binding protein I (CRBP1) is an essential component of the retinoic acid pathway (Ghyselinck et al. 1999). CRBP1 is an intracellular carrier protein that is responsible for transporting retinol for storage in the cell. Several significant findings indicate that CRBP1 is an important gene in breast cancer. CRBP1 expression is down regulated in 24% of human breast cancers (Kuppumbatti et al. 2000) and CRBP1 silencing through promoter methylation is a common epigenetic event in the progression of breast cancer (Esteller et al. 2002). Additionally, CRBP1 warrants further investigation due to a number of studies indicating its role in regulating cell differentiation, anchorage-independent growth, and tumor suppression (Farias et al. 2005). A microarray study examining transcriptional changes within the mammary gland in response to estrogen and progesterone levels mimicking pregnancy identified CRBP-1 to be upregulated (Lu et al. 2008). All of this evidence points to the important role of CRBP-1 in breast cancer and supports our investigation into its role in mammary epithelial cells.

We propose that changes to CRBP1 expression play an important role in breast cancer development and that understanding the role of CRBP1 may be vital to developing preventative therapies. Utilizing mouse models, we will examine how pregnancy affects CRBP1 expression and how loss of CRBP1 impacts cell proliferation and apoptosis in the

mammary gland. In vitro studies using immortalized human mammary epithelial cells treated with various growth enhancing and growth inhibitory reagents will provide an examination of CRBP1 expression patterns in breast tissue. Finally, methylation analysis of human mammary epithelial cells donated by patients at a local hospital will provide evidence about how age and parity impact promoter hypermethylation of several genes, including CRBP1.

CHAPTER 2

CRBP1 IS PERSISTENTLY UPREGULATED IN RESPONSE TO PREGNANCY

Introduction

The correlation between early pregnancy and decreased risk of developing breast cancer is well documented in humans and is reproducible in animal models. Although the molecular mechanisms responsible for this effect are unascertained, it has been shown that pregnancy hormone levels of estrogen and progesterone result in the sensitization of the tumor suppressor gene, p53, to DNA damage in rodents (Sivaraman et al. 2001, Minter et al. 2002). It has also been documented that human mammary epithelial cell treatment with retinoids results in the upregulation of p53 in vitro (Zhang et al. 2005). Furthermore, retinoids have been shown to have potent chemopreventive abilities against breast tumorigenesis in rodents (Wu et al. 2000). Taken together, these observations about p53 suggest a potential relationship between parity-induced protection and the retinoic acid pathway.

As a DNA damage detection gene, p53 plays an important role in DNA damage repair and is capable of inducing the apoptosis cascade. Breast cancer develops from an accumulation of mutations and DNA damage that eventually lead to a loss of cell cycle control. Given the important role that p53 plays in DNA damage repair and programmed cell death, it is clear that p53 is a vital tumor suppression gene. We propose that identifying other genes that are transcriptionally upregulated in response to pregnancy

and are involved in p53 signaling pathways could provide important insight into the parity-induced protection mechanism.

In an effort to further characterize the molecular effects of pregnancy, researchers have developed the means to artificially replicate the effects of pregnancy through the administration of hormones. Doses of estrogen and progesterone that produce circulating levels of hormones equivalent to those found in mid-pregnancy replicate the effects of pregnancy with regard to protection against carcinogen-induced breast cancer in cell culture and rodent models (Guzman et al. 1999). Utilizing this method, a microarray study was conducted in D. Joseph Jerry's laboratory to show transcriptional gene changes in response to pregnancy-level estrogen and progesterone administration. The results of the microarray identified CRBP1 as a gene that is upregulated in response to estrogen and progesterone exposure (Lu et al. 2008). Based on this finding, it is known that CRBP1 transcription can be stimulated by pregnancy hormones *in vitro*, but this effect has yet to be confirmed in vivo.

We propose that CRBP1 expression will be persistently changed in response to pregnancy in vivo. Through the use of a mouse pregnancy study, we will examine CRBP1 expression through PCR analysis and also CRBP1 translational changes using immunohistochemistry. This experiment will prove that the effect of pregnancy hormones on CRBP1 expression *in vitro* can be replicated in a mouse model in vivo and will further our evidence that CRBP1 plays an important role in pregnancy-induced protection against breast cancer.

Materials and Methods

Animal Husbandry & Surgery

12- 8-week-old female nulliparous BALB-c mice were allowed to become pregnant or not. Mice underwent a gestation period of three weeks, birthed their pups, pups nursed for three weeks and were then weaned. The mammary glands of the female mice were allowed to involute for two weeks. Mice were then euthanized at 16 weeks of age using carbon dioxide inhalation. The 4th inguinal mammary glands were removed from the left side and paraffin-embedded for immunohistological staining. The 4th inguinal mammary glands from the right side were flash frozen using liquid nitrogen for RNA isolation (Fig 2.1).

RNA Isolation

Mouse mammary glands harvested for RNA isolation were weighed and averaged between 50-80 mg. 1 mL of TRIZOL® (Invitrogen) reagent was added to each gland and the tissue was homogenized. Samples were centrifuged at 12 X g for 15 minutes, supernatant was removed, and fat was discarded. 0.2 mL of chloroform was added to each sample, samples were incubated at room temperature for 3 minutes, and then centrifuged at 4°C at 12 x g for 15 minutes. Following centrifugation, the aqueous phase was removed and the RNA was precipitated using 0.5 mL of isopropyl alcohol. After a brief incubation period, samples were centrifuged again at 4°C. After centrifugation, the supernatant was removed from the samples and the RNA pellet was washed using 1 mL of 75% ethanol. Samples were again centrifuged at 4°C at 7500 x g for 5 minutes. The RNA pellet was briefly dried and the RNA was resuspended in RNase free water. RNA

quality was ensured by spectroscopy and samples were diluted to 100ng/μl and stored at -20°C for future use.

Quantitative PCR

PCR reactions to measure CRBP1 expression in mouse DNA isolated from the experiment were conducted using an Mx4000 Multiplex Quantitative PCR system (Stratagene). CRBP1 primers utilized were F 5^1 AGTGGATGGTGGAAGAAAC 3^1 and R 5^1 CCCAGGCCTGTGTAAGGTA 3^1 and GAPDH was used as a control for the experiment with primers F 5^1 TTCACCACCATCGAGAAGGC 3^1 and R 5^1 GGCATGGACTGTGGTCATGA 3^1. 1 μL of DNA was amplified in a 10 μL reaction mixture containing 2.4 μL of Mastermix (Brilliant® SYBR® Green QPCR Mater Mix, Stratagene), 2.4 μL of DNase fee water, 0.6 μL each of 12.5nM primers, and 0.4 μL of RT. Each PCR product was amplified over 40 cycles at an annealing temperature of 55°C. Included with each PCR were a series of controls.

Immunohistochemistry

All immunohistochemical studies were performed using the Dakocytomation LSAB® System-HRP kit (Dako, Denmark). Formalin-fixed, paraffin embedded mammary tissue was cut on a Leica microtome at a thickness of 4μm on Superfrost plus slides. Slides were dried by microwave for 1 minute and then in a 62°F oven for 15 minutes. Slides were then deparaffinized three times with xylene, cleared with graded ETOH (100% X2, 95%, 70%), and rinsed in DIH_20. The EZ-Retriever® system (BioGenex, San Ramon CA) for antigen retrieval was used with Antigen Retrieval Citra Solution 10X (BioGenex, San Ramon CA)(citrate buffer pH 6.0) and was brought to a

working 1X dilution with DIH_2O. Slides were allowed to cool for 20 minutes then rinsed in DIH_2O and placed in TBS buffer with Polysorbate 20 (2.5 mL to 5 liters of TBS). Slides were then loaded on the DakoCytomation Autostainer (Dako, Denmark) and stained according to the manufacturer description. The primary antibody used was a polyclonal antibody to CRBP or Actin with a secondary anti-rabbit antibody (Dako polymer detection kit). Sections were then stained with diaminobenzidine (DAB) chromogen for 7 minutes. The slides were then counterstained for 15 seconds in Mayer's hematoxylin, then washed in glacial acetic acid water for 15 seconds, and washed in ammonia water to blue. Finally, slides were dehydrated in ETOH and xylene before manual coverslipping.

Results

In this experiment, we proposed to examine persistent changes in CRBP1 gene expression as a result of pregnancy. RNA was isolated from parous and nulliparous mouse mammary glands and quantitative PCR was used to examine changes in CRBP1 transcription levels. Upon comparison of the two experimental groups, we found that CRBP1 expression was significantly upregulated in the parous mice (Fig 2.2). The parous mouse mammary glands showed a significant increase in CRBP1 transcription of approximately 4 times the relative fold induction of the nulliparous mice. The nulliparous mouse RNA showed no significant change in CRBP1 expression. This result confirmed that CRBP1 expression was upregulated at the transcriptional level in response to pregnancy *in vivo*.

After discovering the transcriptional increase in CRBP1, we wanted to examine changes to gene expression at the protein level. Using the parous and nulliparous mouse mammary glands that were paraffin-embedded, we utilized an antibody for CRBP to examine expression and distribution of the CRBP protein. The parous mouse mammary glands showed a significant amount of CRBP staining in the myoepithelial and the luminal epithelial cells while the nulliparous glands only expressed CRBP in the myoepithelial cells (Fig 2.3). Of note, was the unexpected observation that CRBP was found in the nucleus as well as the cytoplasm. Immunohistochemical staining of mammary glands from CRBP1 knock out animals confirmed that our antibodies were specific for CRBP1. This assay confirmed that CRBP1 was persistently upregulated in the parous mouse mammary gland even after two weeks of involution.

Discussion

The molecular mechanisms responsible for parity-induced protection are still under investigation. Based on a key microarray study mimicking the effects of pregnancy that showed CRBP1 upregulation in response to increased levels of estrogen and progesterone, we proposed that CRBP1 may play an important role in parity-induced protection and should be altered in response to pregnancy. In this study, we confirmed that CRBP1 transcription is persistently upregulated in response to pregnancy *in vivo* and that CRBP1 translational changes are also observed.

One of the most significant findings of this experiment was the persistent upregulation of CRBP1 transcription after mammary gland involution. Postlactational involution is an important remodeling process the breast undergoes after lactation

involving cell death via apoptosis. There is a growing body of literature suggesting that pregnancy and the breast remodeling that occurs during involution plays a significant role in reducing long-term breast cancer risk (Radisky et al, 2009). Interestingly, p53 mediates the apoptosis observed during involution and becomes primed to respond to future carcinogenic challenges after pregnancy (Jerry et al, 1999). The observation that CRBP1 continues to be upregulated even two weeks after weaning suggests that pregnancy relays a permanent effect on its expression in the mammary gland, similar to published findings about p53. This finding adds support to our hypothesis that CRBP1 plays an important role in parity-induced protection and further suggests a potential relationship between CRBP1 and p53.

The other significant finding of this experiment was the localization change of CRBP1 protein expression in the mammary gland cells. Even with an observed transcriptional change, not all genes are also impacted at the protein level, so we were intrigued when the immunohistochemical analysis showed new expression of CRBP1 in the parous mammary gland. As was observed in the nulliparous mice, prior to pregnancy CRBP1 is localized in the myoepithelial cells and is not found in the epithelial or cytoplasm. After pregnancy and involution, CRBP1 expression was located in the epithelial cells and cytoplasm. This new localization of CRBP1 is an important observation and warrants further investigation in the future.

One challenge we initially experienced with this experiment was demonstrating the specificity of our CRBP antibody. CRBP exists in several forms in the body, CRBP1 being just one of them. After immunohistochemistry, we observed that CRBP expression in the parous mice was found in the epithelium and cytoplasm, in contrast to the

myoepithelial CRBP expression in the nulliparous mice, but we were unable at that time to prove that is was specifically CRBP1 expression we were visualizing. In order to prove the specificity of our antibody, we later utilized CRBP1-null mice and repeated the experiment. We were able to demonstrate that our antibody was specific for CRBP1, strengthening the significance of this experiment.

This experiment provided important information about the impact of pregnancy on CRBP1 expression. We were able to confirm that CRBP1 expression, both at the transcription and translation levels, is permanently altered in response to pregnancy in vivo. Based on this experiment, we can say that CRBP1 expression is temporally expressed at the correct time to play an important role in parity-induced protection against breast cancer and propose to continue characterizing the role of this gene.

Figure 2.1 Experiment Overview

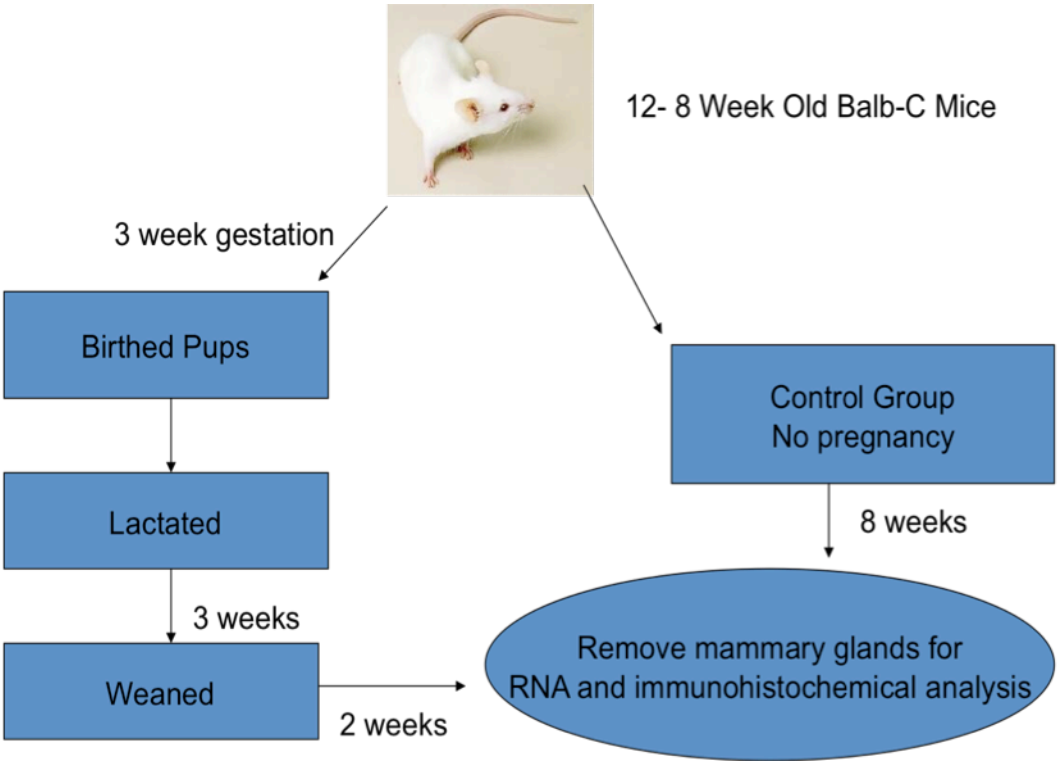


Figure 2.2 qPCR gene expression results for CRBP1

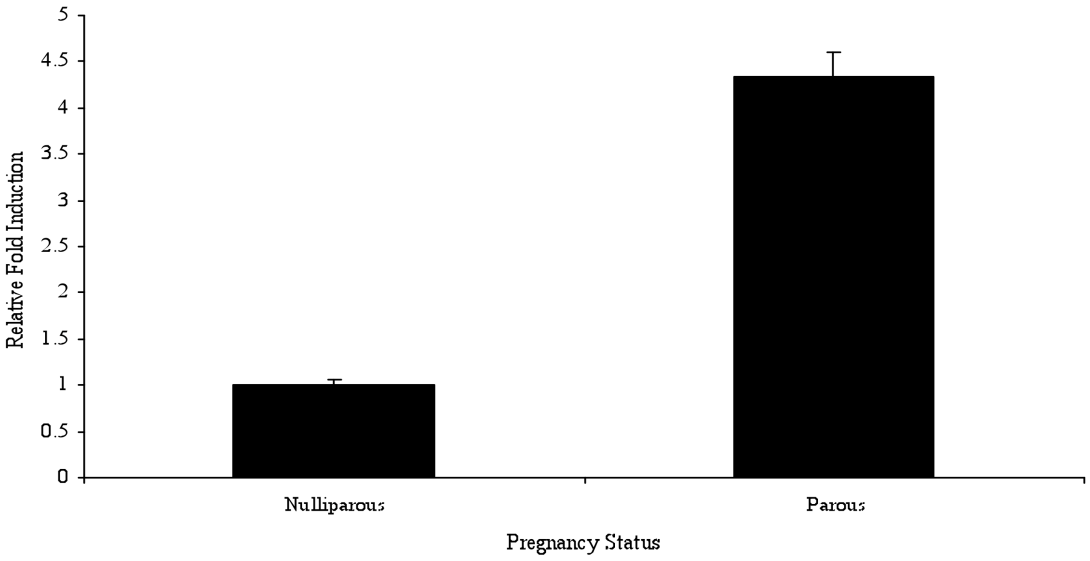
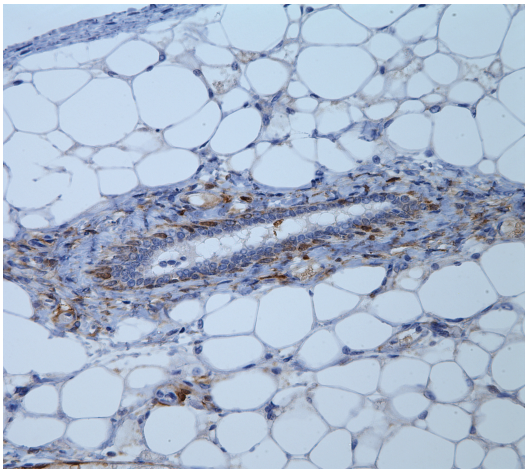
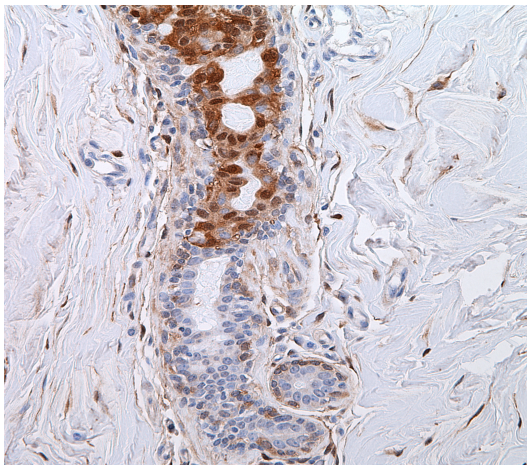


Figure 2.3 Immunohistochemical staining of mouse mammary glands for CRBP1



Nulliparous Mammary Gland



Parous Mammary Gland

CHAPTER 3

LOSS OF CRBP1 DOES NOT EFFECT CELLULAR APOPTOSIS IN RESPONSE TO RADIATION INDUCED DNA DAMAGE

Introduction

As was demonstrated in the previous experiment, CRBP1 expression is persistently upregulated in response to pregnancy in the mouse mammary gland. Although these results indicate a change to CRBP1 expression in response to parity, they do not explain if this change contributes to the phenomenon of parity-induced protection and, if it does, by what mechanism. In this experiment, we propose to further investigate how CRBP1 may be contributing to parity-induced protection against breast cancer, specifically by upregulating the expression or sensitization of p53.

P53 is a DNA damage repair gene and an important tumor suppressor. P53 is a known activator of programmed cell death, or apoptosis, both during development and in response to irreparable DNA damage. Loss of p53 expression has been shown to be an essential part of tumorigenesis and restoration of the gene leads to tumor regression *in vivo* (Ventura et al. 2007). Thus, loss of p53 expression, which is common in many types of cancer, prevents the apoptotic cascade from being properly activated and allows cells with DNA damage cells to replicate.

Treatment of 76N tert human mammary epithelial cells with retinoids has been shown to increase the expression p53 in vitro (Zhang et al. 2005). It has also been established that p53 is sensitized to DNA damage during pregnancy (Lu, et al. 2008, Tu, et al. 2005, Sivaraman et al. 2001). When comparing p53 and CRBP1 regulation they

both have been shown to be positively regulated in response to pregnancy or hormonal treatments mimicking pregnancy. P53 is a downstream target of the retinoic pathway of which CRBP1 is a member. Based on this data, it is possible that there is a relationship between CRBP1 upregulation and persistent expression in response to pregnancy and the sensitization of p53 to DNA damage during pregnancy. We hypothesize that CRBP1 expression is vital to p53 sensitization in response to pregnancy and that loss of CRBP1 expression will result in p53 have a decreased apoptotic response to radiation-induced DNA damage.

One useful technique to investigate the relationship between two genes is to eliminate the expression of the upstream gene and to see how this loss affects its downstream gene target. In order to accomplish this in our experiment, we chose to utilize transgenic mice created in the lab of Pierre Chambon lacking CRBP1 (Quadro et al. 1999). Utilizing the CRBP1 knock-out mice and wild type mice of the same background, we propose to examine if the loss of CRBP1 has an effect on p53 and its DNA damage response in the cell.

One mechanism for cellular death is apoptosis or programmed cell death. When a cell's DNA is damaged beyond repair, the apoptotic cascade, which includes endogenous endonucleases, is activated to dispose of it. The endonuclease is responsible for cleaving the DNA into oligonucleosomes generating free 3'-OH groups at the end of each fragment. A TUNEL assay labels the free hydroxyl groups with fluorescein-conjugated deoxynucleotides. When excited, the fluorescein generates a fluorescent signal that can be detected using fluorescence microscopy. Cells that are fluorescent are then counted as TUNEL-positive and undergoing apoptosis. In this experiment, we plan to use a TUNEL

assay to measure the percentage differences of TUNEL-positive cells between CRBP1 null and wild-type mice and extrapolate that finding to an understanding of the relationship between CRBP1 and p53.

CRBP1 expression is often lost during the progression of breast cancer. *In vitro* studies have suggested the CRBP1 is involved in cellular differentiation and that it may play a role in ductal epithelial cells susceptibility to cell death (Farias et al. 2005, Kuppumbatti et al. 2001). Thus the loss of expression may impact the ability of the mammary gland to remove unwanted cells and increase risk of breast cancer. We would like to see if the loss of CRBP1 expression affects the expression or activity of the tumor suppressor p53 and whether there is a reduction in cellular death as a result.

Materials and Methods

Animal Husbandry

CRBP1 knockout C57 BL/6.129 mice were created in the laboratory of Pierre Chambon (University of Strassbourg) and were provided to us by William Blaner (Columbia University). Mice wild-type for CRBP1 were also obtained with a C57 BL/6.129 background. Mice were backcrossed three times to BALB/c mice to ensure a susceptible background to breast cancer. Mouse genotypes were confirmed throughout the experiment to ensure that the knockout mice truly lacked CRBP1.

Experimental Procedures and Surgery

As is shown in Figure 3.1, 6-week old BALB/c-C57 BL6.129 mice were divided into four experimental groups with 9 mice per group: wild-type nulliparous, wild-type

parous, CRBP1 knockout nulliparous, and CRBP1 knockout parous. Mice were then impregnated or not. After a full cycle of pregnancy, 1 week of lactation, and three weeks of involution, mice were treated 5Gy of whole body radiation and then put back into their cages for 6 hours to allow DNA damage and cellular death to occur. Mice were then euthanized using carbon dioxide inhalation. The 4^{th} inguinal mammary glands of the mice were then removed and paraffin imbedded for immunohistochemical analysis and TUNEL assay. Spleens from one mouse in each experimental group were also removed to confirm that the whole-body radiation was successful in inducing DNA damage.

TUNEL Assay

For our TUNEL assay, we used the Calbiochem® Fluorescein-FragEL (Fragment End Labeling)™ DNA Fragmentation Detection Kit. Kit procedures for conducting the assay were followed. Completed slides were labeled with the genotype and parity status of the mouse as well as their number. Slides were stored at 20°C when not been examined or photographed.

Fluorescent Microscopy and Cell Counting

After completing the TUNEL assay, slides were examined for apoptotic cells using a Nikon Eclipse TE2000-U™ and Nikon X-Cite Series 120™ microscope system for fluorescence and Metavue™ to view and manipulate the images. Each animal was represented by one slide. For each slide, 5 mammary ducts were randomly chosen using DAPI only. After selecting a duct, a picture was taken with DAPI alone, TUNEL alone, and then the two images were overlaid to reveal positive TUNEL staining (Figure 3.2). Positive epithelial TUNEL cells were then counted for each duct as well as the total

number of epithelial cells. Statistical analysis was then conducted using Microsoft Excel to determine the percentage of TUNEL-positive cells in each of the experimental groups (Figure 3.3).

Results

CRBP1 is not required for parity-induced p53 sensitization to DNA damage and activation of the apoptotic cascade (Figure 3.4). Utilizing CRBP1 knock-out mice and wild-type mice of the same background, we conducted a pregnancy study, inducing a DNA damage response, and conducted a TUNEL assay to examine changes in apoptotic death. Upon examination of the TUNEL-positive percentages for each experimental group, there was no significant difference between the CRBP1 null parous mice and the CRBP1 wild-type mice. This finding suggests that CRBP1 does not play an essential role in p53's ability to respond to DNA damage and induce apoptosis.

Discussion

This study definitively demonstrated that CRBP1 expression is not required for p53 sensitization during pregnancy and its response to radiation-induced DNA damage. The results of our experiment showed that there was no statistical difference in the amount of apoptosis between the wild-type parous mice and those with those lacking CRBP1. Although our hypothesis that CRBP1 played an important role in priming p53 as part of its role in parity-induced protection against breast cancer was not confirmed in this experiment, we were able to rule out one of the ways that CRBP1 could contribute to tumor suppression.

There were two major successes of this experiment: backcrossing the wild-type and knockout mice and accomplishing the TUNEL assay using the mouse tissue. One of the initial challenges we encountered in this experiment was back-crossing the knock-out and wild type mice C57 BL/6.129 mice onto a BALB/c background. This was an important step in our experimental design as the BALB/C background is more susceptible to mammary carcinogenesis. We backcrossed the mice three times before beginning our experiment. Initially, the knockout mice had challenges breeding and, when they did, had low litter counts. Eventually, once they had been successfully backcrossed twice, the breeding issues resolved themselves, but it did slow the experiment. It was also important that we confirm that the backcrossed null animals were in fact lacking CRBP1. Using PCR analysis, we did confirm prior to setting up the experimental groups that all of the animals were in fact CRBP1 null or CRBP1 wild-type and later further confirmed this at the protein level using immunohistochemistry (Figure 3.4). Additionally, because we had fewer knockout breeding pairs than wild-types, we had to conduct the experiment in two waves, with 4 mice in each group the first, and 5 in the second. Although the experimental procedures for the two sets were identical, it would have been preferable to have had all of the animals in one set in order to ensure that no environmental factors affected them.

The other major success of this experiment was getting the TUNEL assay to work with the mouse mammary gland tissue. Although this assay was conducted using a kit (Calbiochem Frag-El), TUNEL assays can be challenging with certain tissues. We were able to observe apoptotic cells in every experimental group and confirm that the assay did work properly. Our death counts were higher in the parous mice versus nulliparous mice

which is in agreement with the literature, but our overall percentages of TUNEL-positive cells in the parous animals were lower than the literature. We think this could be due to the fact that we only completed 3 backcrosses on the susceptible mouse background and that we probably should have done 6 backcrosses to be certain that the majority of the genes were coming from the BALB/c strain.

A challenge we experienced in this study was examining p53 expression using immunohistochemistry. Initially, we set out to conduct the TUNEL assay to compare the amount of apoptotic activity between parous CRBP1 null and wild-type animals, but we also planned to utilize a p53 specific-antibody to examine protein-level changes in gene expression. Unfortunately, we were unable to complete this part of the experiment because we were unable to obtain a working p53 antibody. This piece of data would have been helpful in understanding the TUNEL assay results as we can only extrapolate from the TUNEL results that CRBP1 loss did not affect p53 regulation, but it would have been beneficial to have confirmed this using immunohistochemistry.

Based on the results of this experiment, CRBP1 is not required for p53-mediated apoptosis in response to radiation-induced DNA damage. Although this finding may be accurate, there two ways that this experiment could be improved upon in the future to ensure that our finding is correct. Both CRBP1 and p53 are known to be regulated by estrogen. In vivo, the female mouse is undergoing her own estrous cycle, but we did not account for this in our experimental design. If this experiment is to be repeated, the timing of the estrous cycle of the individual mice in the study should be better controlled for utilizing regular blood tests. This would help to help to eliminate one possible source of error. Additionally, one of the challenges we experienced in conducting the TUNEL

analysis was uniformity within the mouse mammary ducts in terms of size. Since only one slide was prepared per mouse, there were animals that only had very small or very large ducts, which potentially could have impacted our statistical analysis. In the future, at least two slides, preferably from different depths in the paraffin-imbedded gland or from different glands should be utilized to control for such error.

Figure 3.1 Experiment Overview

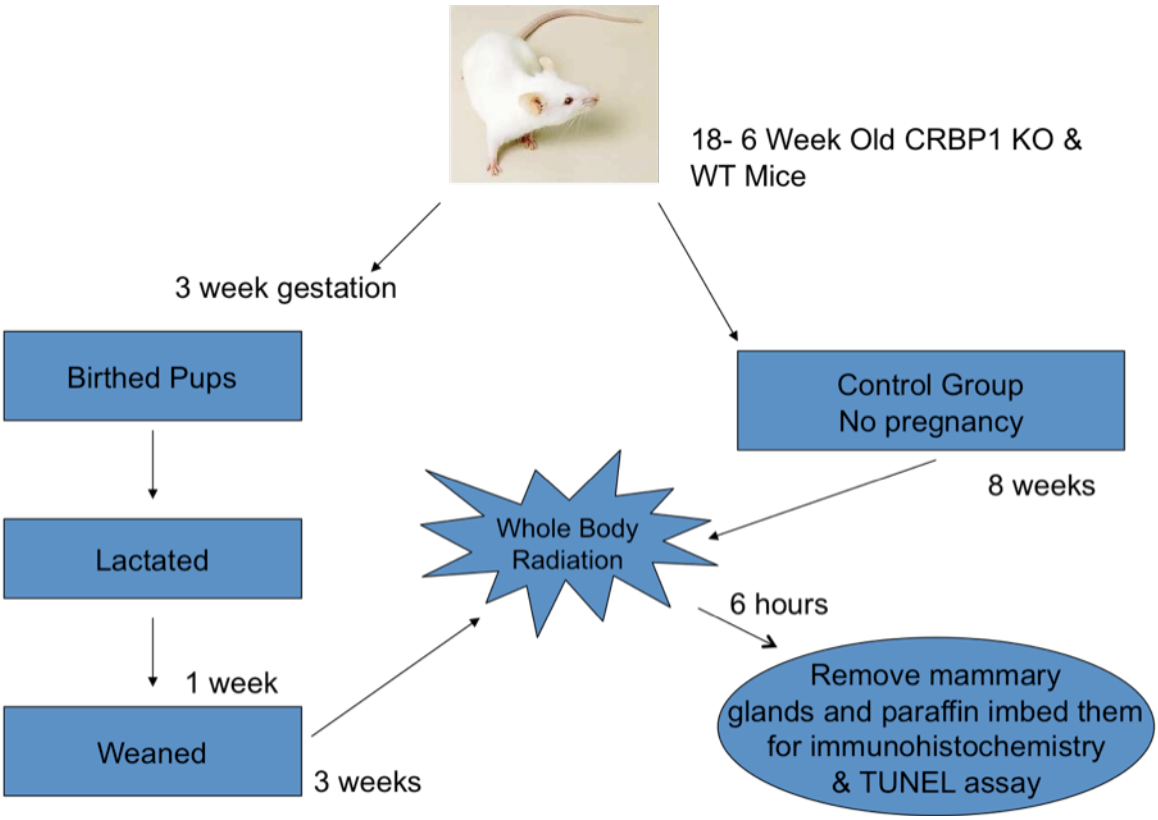
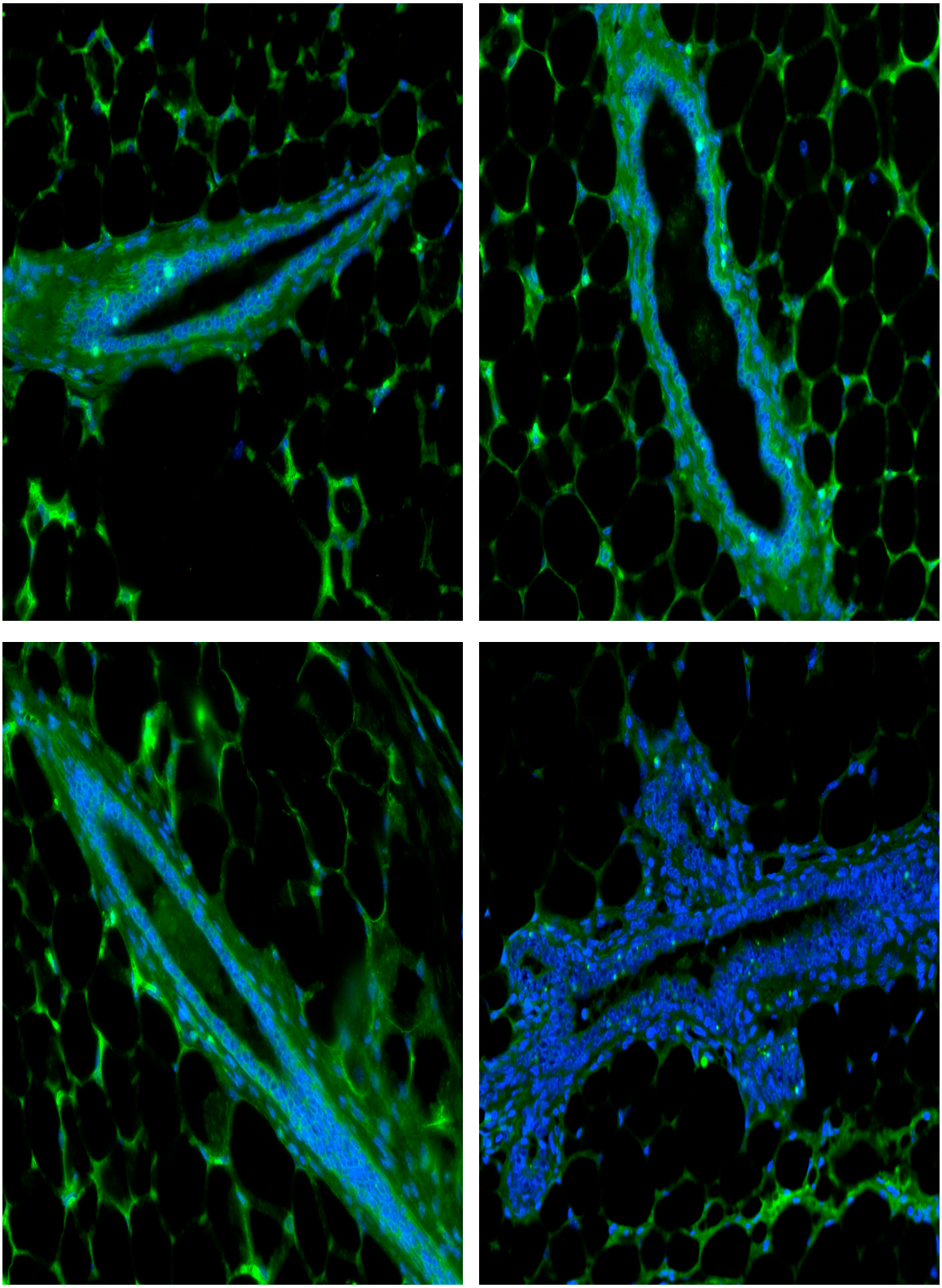


Figure 3.2 Sample of TUNEL pictures



Top row is TUNEL (+) parous and nulliparous mice and bottom row is TUNEL (-) parous and nulliparous mice. Positive cells are azure in color.

Figure 3.3 Percent of TUNEL-positive cells for each experimental group

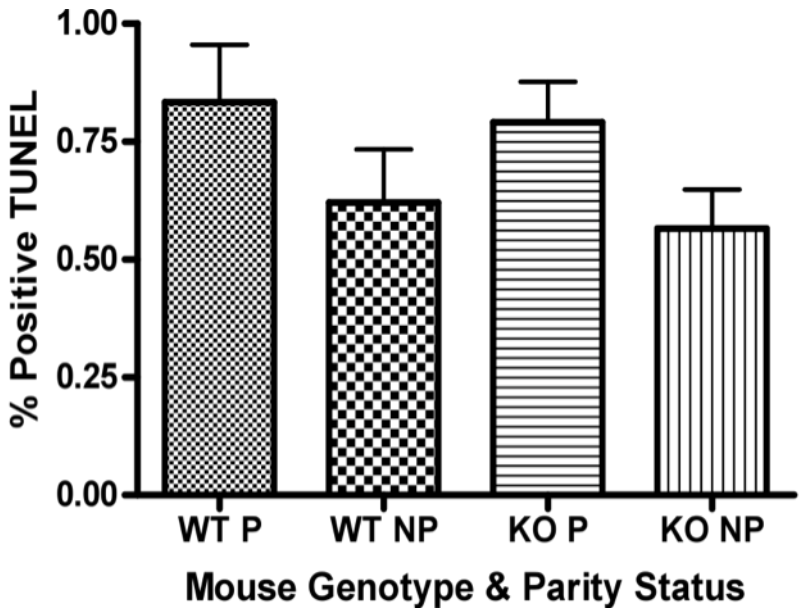
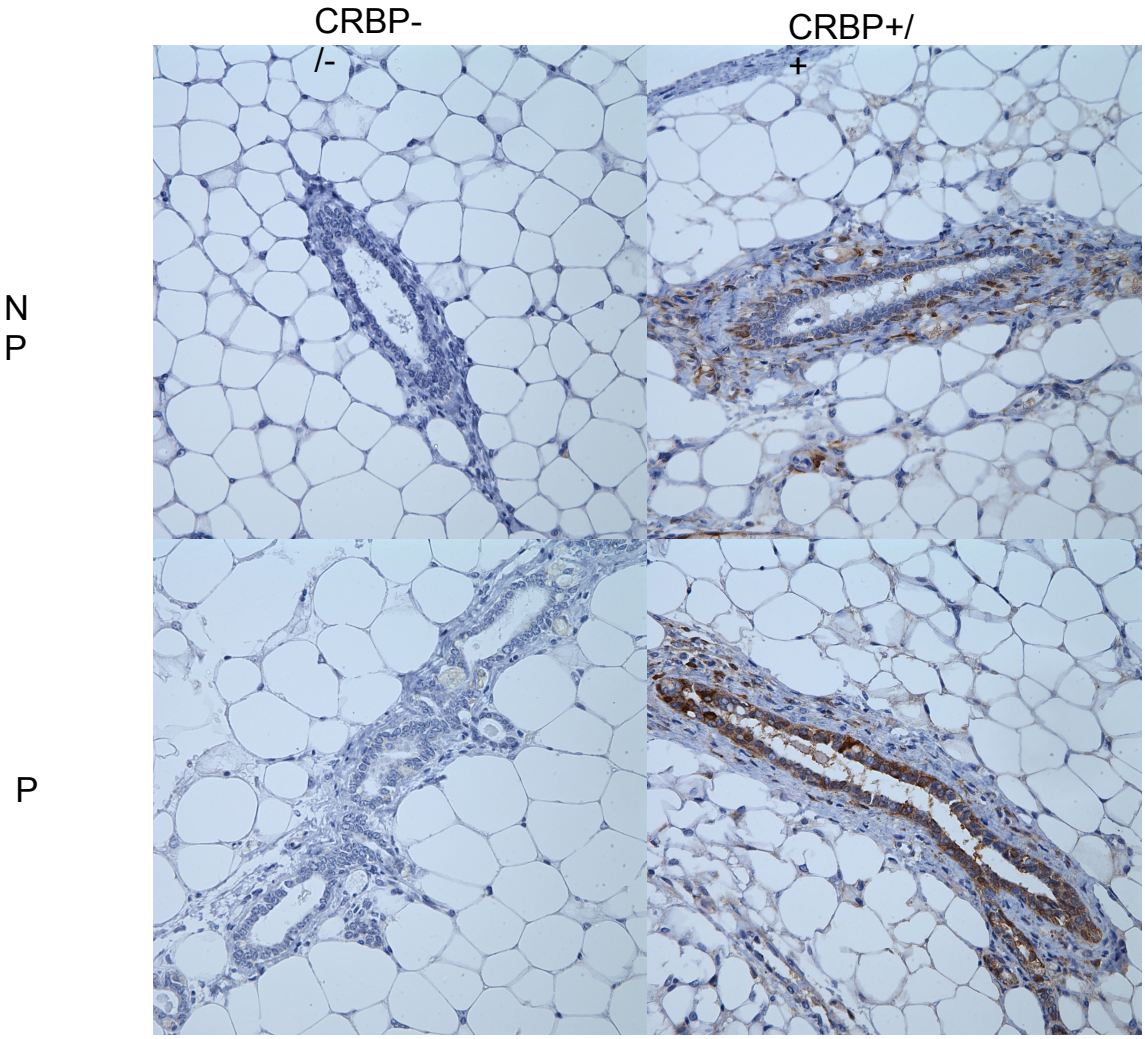


Figure 3.4 Immunohistochemical staining for CRBP1 expression in CRBP1 knockout and wild-type mice, parous and nulliparous



Staining confirms that CRBP1 null mice do not express CRBP1 protein.

CHAPTER 4

CRBP1 IS REGULATED BY AGENTS WHICH INHIBIT AND PROMOTE GROWTH

Introduction

The role of CRBP1 in pregnancy-induced protection against breast cancer is still under investigation, but research has uncovered clues about the important role CRBP1 plays in the cell. Studies have demonstrated that retinoids are important for proper cell growth and differentiation and that treatment of MCF-7 breast cancer cells in vitro with retinoic acid can induce cell cycle arrest (Tighe and Talmage 2004, Zhu et al. 1997). In addition to being a critical player in the retinoic acid pathway, CRBP1 has independently been implicated in cell growth regulation and tumor suppression. Specifically, CRBP1 has been shown to play a role in anchorage-independent growth, cell differentiation, and cell proliferation (Kuppumbatti et al. 2001, Farias et al. 2005). One of the key steps in cancer progression is the loss of cell cycle regulation and, consequently, uncontrolled growth. Retinoids have already been shown to have tumor suppressive activity through growth regulation, and a better understanding of how CRBP1 is regulated in the cell may strengthen our understanding of its role in controlling cellular growth and tumor suppression.

There are many natural and synthetic chemical compounds that have been previously shown to regulate growth in the cell. We propose that by treating immortalized human mammary epithelial cells with chemical agents whose effects on cell growth are known, we can determine if CRBP1 expression is altered in response their activity. We hypothesize that if CRBP1 regulates cell proliferation in human mammary

epithelial cells then compounds that are known to effect cell growth may regulate its expression. In order to select appropriate treatments that would contribute to the overall understanding of CRBP1 and cell regulation, we chose chemical agents that have been previously shown to regulate CRBP1 as controls and agents whose effects on CRBP1 are unknown, but have been shown to work through receptors and pathways CRBP1 is known to interact with.

This experiment will allow us to examine if growth inhibitory agents regulate CRBP-1. We would like to examine the expression of CRBP1 in immortal mammary epithelial cells in response to 9-cis retinoic acid, TGFb, IGFBP3, and Tamoxifen. These agents have all been shown to inhibit growth or act in a preventive fashion. As a known downstream target of the retinoic acid pathway, we expect that CRBP-1 will be upregulated in response to retinoic acid treatment in epithelial cells. In fibroblasts, it has been shown that CRBP1 is regulated by TGFβ, transforming growth-factor beta, and it is has also been shown that retinoids act, in part, by activating TGFβ (Xu et al. 2001, Kojima et al. 1993). Based on these observations, we propose to test whether TGFβ alone is sufficient to upregulate CRBP1 expression in human mammary epithelial cells. IGFBP3, insulin-like growth factor binding protein 3, is a downstream gene target of TGFβ and is also been shown to increase TGFβ signaling (Schedlich et al. 2002). Furthermore, it inhibits IGF-1 induced signaling, a gene known to increase cell proliferation. We would like to test the idea that IGFBP3 will increase CRBP1 expression. In the uterus, we have previously observed that CRBP-1 is upregulated in response to Tamoxifen, a potent cancer drug often used in the treatment of estrogen-receptor positive breast cancer. However, the uterine cells can have very different

responses to Tamoxifen than mammary epithelial cells. We would like to determine whether Tamoxifen will increase CRBP1 expression in the mammary epithelial cells.

In contrast, we expect to see that CRBP-1 expression is abrogated by growth inducing agents. IGF-1, insulin-like growth factor 1, is known to promote cellular proliferation. We have previously noted that IGF-1 inhibits estrogen and progesterone induced CRBP1 expression and plan to confirm this finding using the mammary epithelial cells in culture. Insulin is known to activate the IGF receptor consequently promoting cellular growth. In this experiment we would like to whether treating cells with physiological levels of insulin alone is sufficient to block CRBP1 expression.

Materials and Methods

Cell Culture

76N tert, immortalized human mammary epithelial cells, were plated in 6-well plates at a density of 80,000 cells per well in 3mL of 76N tert growth media. Cells were grown overnight at 37°C in a humidified incubation chamber with 5% CO_2.

Cell Treatment

After approximately 24 hours of growth, the 76N tert growth medium was removed, cells were rinsed with PBS, and 3 mL of DMEM-F12 plus antibiotic supplemented with the appropriate chemical agent was added to each well. Reagents included 9-cis retinoic acid, estrogen and progesterone (combination treatment), IGF-1, Tamoxifen, TGFβ, and IGFBP3. Ethanol, DMSO, H_2O, and BSA.HCl were used as controls. Each reagent treatment was performed in triplicate. Reagent concentrations were calculated to imitate

known physiological levels (Table 4.1). Cells were treated overnight and harvested using Trizol® at 24 hours and frozen at -80°C pending RNA isolation. The entire experimental procedure was performed twice to confirm experimental results.

RNA Isolation

Cell lysates suspended in the Trizol® reagent were thawed and 200 μL chloroform was added. Lysates incubated at room temperature for 3 minutes and were then centrifuged at 12 x g for 15 minutes. The aqueous layer was transferred to a new tube and 500 μL of 100% isopropanol was added and samples were then centrifuged. After centrifugation, 1 mL of 75% ethanol was added and samples were allowed to precipitate for at least two hours at -20°C. Samples were then removed from the freezer, centrifuged at 7500 X g for 5 minutes, and liquid was removed from the pellet. RNA pellet was briefly dried and then resuspended in 50 μL of DNase free water, spectroscopy was performed to measure RNA concentration and purity, and samples were diluted to 100 ng/μL.

Quantitative PCR

PCR reactions to measure CRBP1 induction in the 76N tert mammary epithelial cell DNA treated with growth inhibiting and growth promoting agents was conducted using a Mx4000 Multiplex Quantitative PCR system (Stratagene). CRBP1 primers utilized were F 5^1 CGGGTTCGGAACTATATCATGGACTTCTCGA 3^1 and R 5^1 AATTCAAAAATCGGAACTATATCATGGACTTC 3^1 and GAPDH was used as a control for the experiment with primers F 5^1 CCATGGAGAAGGCTGGGG 3^1 and R 5^1 CCAAGTTGTCATGGATGACC 3^1. 1 μL of RNA was amplified in a 10 μL

reaction mixture containing 2.4 μL of Mastermix (Brilliant® SYBR® Green QPCR Mater Mix, Stratagene), 2.4 μL of DNase fee water, 0.6 μL each of 12.5nM primers, and 0.4 μL of RT. Each PCR product was amplified over 40 cycles at an annealing temperature of 55°C. Included with each PCR were a series of controls.

PCR Analysis

In order to analyze the PCR results, each of the experimental runs was broken into three separate PCR runs based on their respective control agents due to the limited sample space for each run. The first PCR panel includes 9-Cis RA, combination estrogen and progesterone treatment, and IGF-1 with ethanol as their controls (Figure 4.1 & 4.2). The second panel includes IGFBP3 with ethanol as its control and insulin with water as its control (Figure 4.3 &4.4). Finally, the third panel includes Tamoxifen with a DMSO control and TGF-β with BSA.HCl as its control (Figure 4.5 &4.6). GraphPad Prism™ was used for statistical analysis and graphing.

Results

CRBP1 is regulated in response to both growth inhibiting and growth enhancing agents. Our experiment confirmed that CRBP1 is significantly upregulated in response to 9-cis RA and TGFβ. In experiment 1, the 9-cis retinoic acid treatment had a great deal of error and was consequently not significant, but in experiment 2 it significantly upregulated CRBP1 expression as we expected (P value 0.0175). This confirms that as a downstream member of the retinoic acid pathway that CRBP1 expression is positively altered in response to retinoic acid. The effect of TGFβ treatment on CRBP1 expression was very significant (P values 0.001) and was confirmed in both experiments. These

highly significant results indicate that TGFβ alone is sufficient to upregulate CRBP1 expression.

This experiment also confirmed that CRBP1 is negatively regulated by IGF-1 in 76N tert cells. We had previously observed that IGF-1 could inhibit estrogen and progesterone upregulation of CRBP-1 and hypothesized that it would decrease CRBP1 expression even in the absence of the hormones. IGF-1 inhibition of CRBP-1 expression was highly significant (P values 0.0042 & 0.0038 respectively) and resulted in approximately a 50% reduction in CRBP1 expression.

Based on the results of our initial mouse experiment and other research, we hypothesized that the combination estrogen and progesterone treatment would significantly upregulate CRBP1 in the human mammary epithelial cells. The results of our PCR analysis did not confirm our hypothesis as CRBP1 was not regulated at all in response to this treatment in either experiment. Error in the experiment was low so we suspect that this result is due to low levels of the estrogen receptor and/or the progesterone receptor. In addition to estrogen and progesterone, insulin also did not have an affect on CRBP1 expression in 76N tert cells.

The results of this experiment were inconclusive for both Tamoxifen and IGFBP3. The results for Tamoxifen in this study were mixed. In experiment 1, treatment with Tamoxifen did not positively regulated CRBP1 expression, but in experiment 2 it did (P value 0.03). These inconclusive results could be the result of treatment error or a number of other factors such as receptor level. IGFBP3 also had mixed effects on CRBP1 expression. In experiment 1, IGFBP3 significantly decreased CRBP1 expression

(P value 0.0090, but this effect was lost in the second experiment. It is important to note however that both IGFBP3 and its ethanol control had large margins of error in the second experiment. The effects of Tamoxifen and IGFBP3 on CRBP1 expression cannot be fully explained by this study and need to be further investigated for more definitive answers.

Discussion

It is clear from the results of this experiment that CRBP1 is regulated by both growth inhibiting and growth enhancing agents. CRBP1 has been implicated in having a role in regulating cell proliferation and has even been suggested to be a tumor suppression gene (Farias et al. 2005). A better understanding of how CRBP1 is regulated provides important clues about the specific mechanisms of how it regulates cell growth and also provides insight into what loss of CRBP1 during cancer progression might cause. This study revealed several important findings that contribute to our knowledge of CRBP1 regulation and also highlights the challenge of replicating physiological conditions in an in vitro cell culture system.

One of the major challenges with conducting a cell treatment experiment is determining how long it takes for the reagent to take affect. Prior to conducting the experiments that are being explored in this chapter, we conducted an initial 24-hour treatment and a 48-hour treatment study with a smaller selection of chemical treatments to determine the ideal treatment time. The PCR results for CRBP1 at both the 24hour and 48 hour time points showed that there was little to no difference between them in

terms of statistical significance and observed trends. Therefore, we chose to utilize the 24-hour treatment window for the larger experiment.

CRBP1 is an integral part of the retinoic acid pathway and is has been previously shown to be upregulated by retinoic acid treatment (Zaitseva et al. 2008). When designing the experiment, we fully expected that 9-Cis RA should significantly upregulate CRBP1 since this gene is a transporter of retinoic acid in the cell. We were initially surprised to find that CRBP1 expression was only significantly increased in one of the experiments (Figure 4.2). Upon closer examination, it is likely that error played a large role in experiment 1 resulting in treatment with RA not being significant (Figure 4.1). Based on these observations, we conclude that CRBP1 is positively regulated by 9-Cis RA in the mammary epithelial cell. Further experiments could be conducted to test the effect of other forms of retinoic acid on CRBP1 expression, such as all-trans RA, and strengthen our finding.

Based on the findings in our mouse study that demonstrated that CRBP-1 is persistently upregulated in response to pregnancy in vivo in the mouse mammary gland, we hypothesized that CRBP-1 would be significantly upregulated in response to the combination treatment of estrogen and progesterone which mimics physiological levels of the hormones that are found during pregnancy in human mammary epithelial cells. After completing the statistical analysis using the PCR data, we found that estrogen and progesterone did not regulate CRBP1 in the 76N tert cells in either experiment (Figures 4.1 &4.2). In fact, there was not even a trend toward regulating CRBP1. In an effort to explain this unexpected result, it is important to consider the differing environments of the cultured cell and the intact mammary gland. In vivo, the mice have their own estrous

cycle controlling internal hormone levels in the mammary gland. As the cultured cells are do not have a regular hormone cycle that is replicated in the cell in vitro. Also, it is possible that the estrogen contained in the 76N tert growth media during the first 24 hours of culture could be confounding our results. Analysis of mouse tissue treated with with estrogen and progesterone (Personal communication from Lesley Mathews) or various estrogen alpha and beta agonists (Personal communication from Eric Roman Perez) support that CRBP1 is positively regulated by estrogen, so this is likely an artifact of tissue culture or a requirement for other cell types present in the mammary gland to provide paracrine signals in response to estrogen.

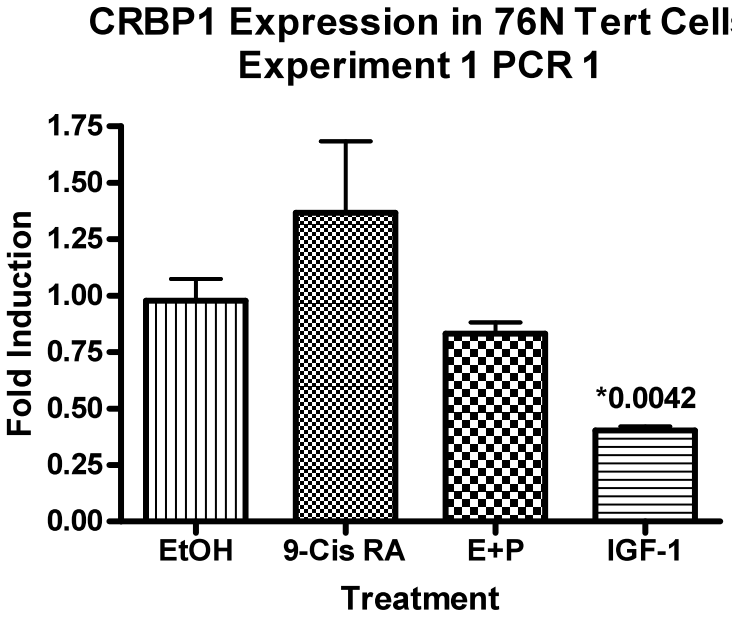
The major finding from this study is that CRBP1 is negatively regulated by growth enhancing agent IGF-1 even in the absence of hormone induction. IGF-1 treatment reduced CRBP1 gene expression significantly in both of the experimental runs with p values of 0.0042 and 0.0038 respectively. IGF-1 has been previously shown to block CRBP1 upregulated expression, but its effects on normal expression levels of CRBP1 were not known. Additionally, it appears that IGF-1 is specifically required to mediate insulin-related effects on CRBP1 as its expression was not impacted by insulin treatment alone.

In conclusion, CRBP1 is regulated by growth enhancers and inhibitors in 76N tert human mammary epithelial cells. Future experiments should focus on reexamining the effects of Tamoxifen and IGFBP3 on CRBP1 expression and should also seek out additional known growth effectors to test and further strengthen what is known about CRBP1 regulation.

Table 4.1 Summary of treatment concentrations used in 76N tert experiments

Summary of Treatment Concentrations Used in 76N tert Experiments		
Treatment	**Stock Concentration**	**Amount used per mL of media**
9-Cis Retinoic Acid	1mM	1µL/mL
Estrogen	1µg/mL	1µL/mL
Progesterone	1mg/mL	1µL/mL
Tamoxifen	1mM	1µL/mL
TGF beta	10 ng/mL	2.5µL/mL
IGF-1	100µg/mL	1µL/mL
Insulin	10mg/mL	2.5µL/mL
IGFBP3	250µg/mL	1µL/mL
EtOH	100%	1µL/mL
DMSO	100%	1µL/mL
BSA.HCl	4mM	2.5µL/mL

Figure 4.1 PCR analysis for CRBP1 expression in 76N tert cells treated *in vitro* with EtOH, 9-cis RA, combined estrogen and progesterone, and IGF-1



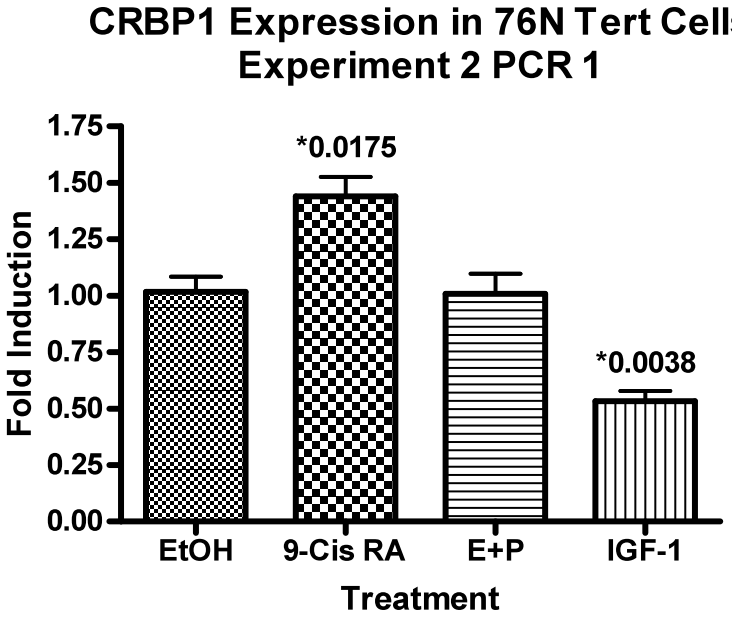
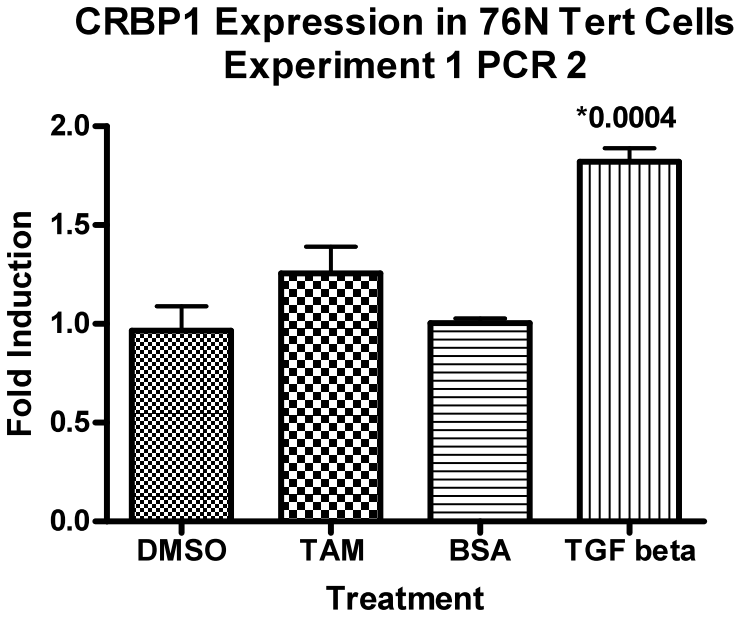


Figure 4.2 PCR analysis for CRBP1 expression in 76N tert cells treated *in vitro* with DMSO, Tamoxifen, BSA.HCl, and TGF beta



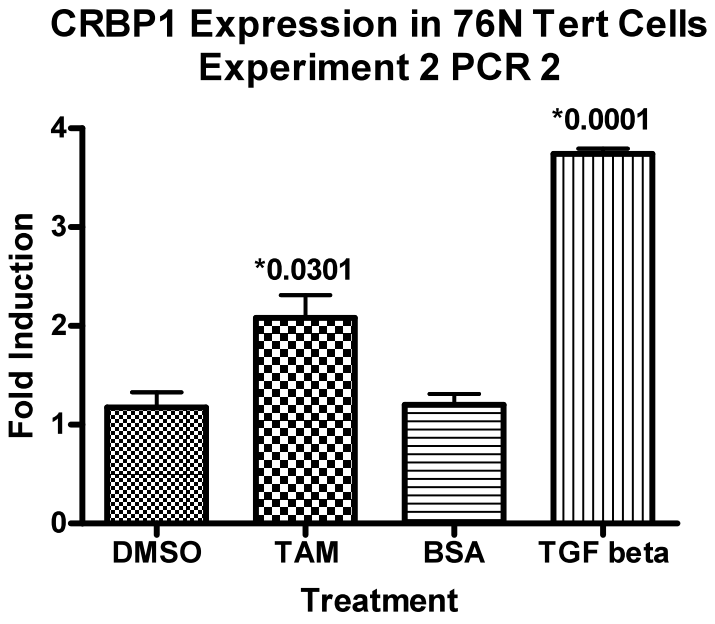
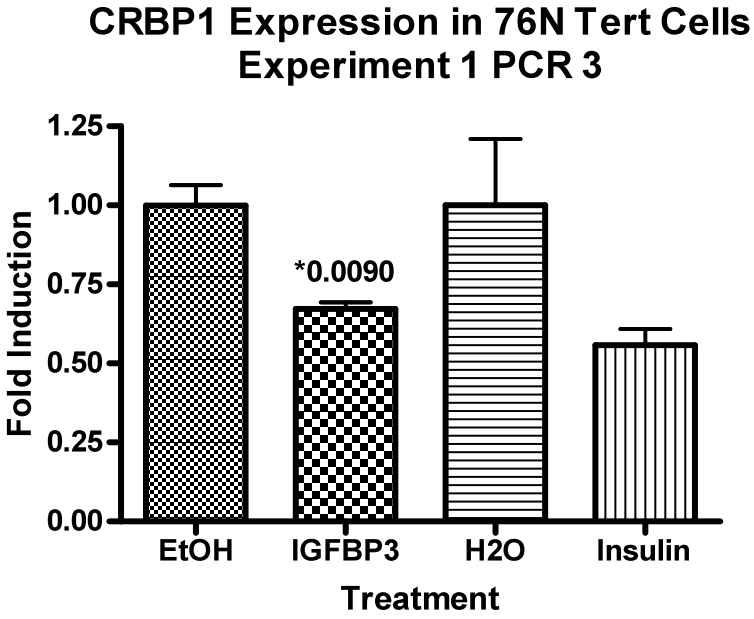
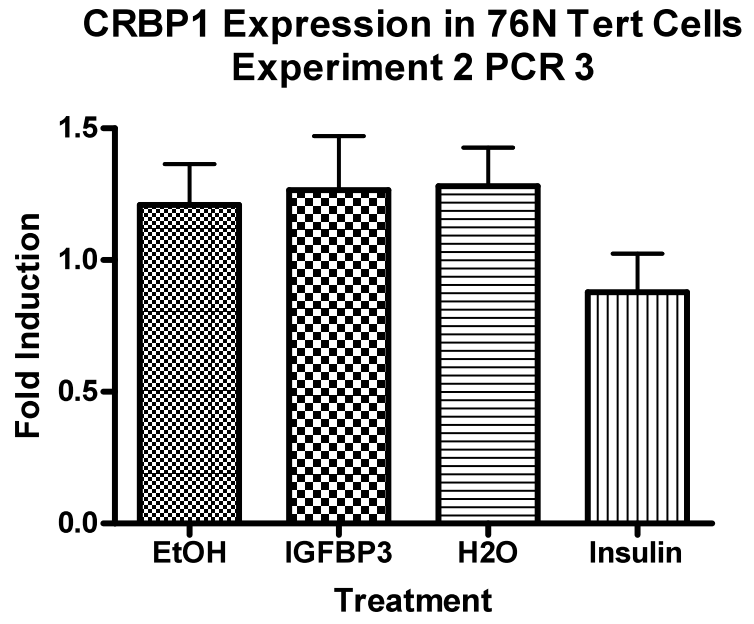


Figure 4.3 PCR analysis for CRBP1 expression in 76N tert cells treated *in vitro* with EtOH, IGFBP3, water, and insulin





CHAPTER 5

EXPLORING THE EFFECTS OF BREAST CANCER RISK FACTORS ON GENE INACTIVATION THROUGH PROMOTER HYPERMETHYLATION

Introduction

Breast cancer arises as the result of a characterized multi-step process that includes the loss of cell cycle regulation and alterations to gene expression. Normally, the body maintains a careful balance of oncogenes and tumor suppressive gene expression, however, in cancer oncogene expression is frequently upregulated while tumor suppressive gene expression is often lost. In recent years, research into the epigenetics of cancer has become a major field of study. One important epigenetic change observed in breast cancer is CpG dinucleotide methylation in the promoter region of specific genes. This aberrant methylation alters the expression of the effected gene and aids cancer growth (Dworkin et al. 2009). We propose that examining changes in promoter methylation in genes commonly altered in breast cancer using benign tissue may provide important insight into the relationship between breast cancer risk factors and promoter methylation.

Epidemiological studies indicate a number of risk factors that may contribute to risk of developing breast cancer for women including age, parity status, and family history (American Cancer Society 2009). Age is an important risk factor for cancer. Specifically, as women age their cells accumulate DNA damage, increasing their likelihood of developing breast cancer (Euhus et al. 2008). Another important risk factor for breast cancer is parity status. It has been well documented that women who have full-term pregnancy before the age of 24 have a lifetime decreased risk of developing the

disease (Russo et al. 2005). This observation is often attributed to changes in gene expression and breast architecture that occur during pregnancy, lactation, and involution. Family history is also an important risk factor for breast cancer. Women with a first degree relative (parent, sibling, or offspring) who have had breast cancer have nearly double the risk of developing the disease and having a secondary relative (aunt/uncle, grandparent, niece/nephew, half sibling) with the disease also increases risk (American Cancer Society 2009). Each of these risk factors can impact a woman's likelihood of developing the disease. We hypothesize that these risk factors may correlate to changes in promoter methylation in three genes whose expression is often altered in the early stages breast cancer development, CRBP1, SFRP1, and RASSF1. For this study, we will compare changes in CpG promoter methylation across these three genes to these risk factors in an effort to determine if there is a relationship between them. We will also examine the individual CpG sites for each gene to determine if any sites are particularly susceptible to methylation.

Epigenetic promoter methylation is a common event early in breast cancer progression. Although there are many genes that are frequently methylated with breast cancer, three such genes, CRBP1, SFRP1, RASSF1, are the subjects of this study. Cellular retinol binding protein is an important gene in the retinoic acid pathway and research has suggested that it may be a tumor suppression gene. CRBP1 hypermethylation has been shown to be the primary mechanism of CRBP1 silencing in human breast cancer and this phenomenon is evolutionarily conserved between humans and mice (Esteller et al. 2002, Arapshian et al. 2004).

SFRP1 belongs to a family of genes known to antagonize the Wnt pathway. Wnt regulates cell proliferation and differentiation and normally binds Frizzled receptor (FZD). SFRP-1 inhibits Wnt signaling by competing with Frizzled for the Wnt binding domain. SFRP1 is located in a chromosomal region that is often deleted in breast cancer and has also been shown to be frequently methylated in patients with the disease (Suzuki et al. 2008). Therefore, loss of SFRP1 gene expression through promoter methylation can result in an overexpression of Wnt leading to unregulated cell proliferation, contributing to the progression of breast cancer.

Finally, RASSF1 is a gene that plays an important role in DNA damage repair and apoptosis. Encoding RAS effector proteins, RASSF1 interacts with DNA repair protein XPA and is required for death receptor-dependent apoptosis. Additionally, RASSF1 inhibits the accumulation of Cyclin D1 in the cell resulting in cell cycle arrest and the inhibition of cell proliferation. Studies have suggested that RASSF1 is an important tumor suppression gene based on its role in DNA damage repair and its frequent hypermethylation in cancer (Hesson et al. 2007).

In order to study to the effects of age, parity, and family history on promoter methylation of our chosen genes, it was necessary to find a source of cells that varied with respect to these variables. We chose to utilize human mammary epithelial cells that were harvested from donated tissue from women undergoing reduction mammoplasty surgery at Baystate Hospital in Springfield, MA. Due to an ongoing study between the Smith Schneider lab and the Jerry lab and the surgical and oncology departments at the hospital, women undergoing breast reduction surgeries at the hospital were propositioned to donate their tissue to cancer research. Mammary epithelial cells were then harvested

and cultured from these tissues and were used in this study. In addition to donating their tissue, women participating in the study were asked various questions about themselves including their age, number of full-term pregnancies, and family history of breast cancer. This epidemiological data was utilized to help us understand the methylation analysis we conducted.

Pyrosequencing has emerged as the preferred method for analyzing DNA methylation (Reed at al. 2009). CpG sites are located in the promoter regions of genes making them a prime target for methylation. DNA methylation results in a methyl group being attached to the cytosine next to the guanine in the CpG island. The first step in methylation analysis is to decipher between cytosines that have been methylated and those that have not. Bisulfite modification of sample DNA converts all unmethylated cytosines to uracil, allowing the methylated cytosines to be easily identified. After bisulfite modification, PCR analysis is used to amplify the DNA for pyrosequencing. Finally, pyrosequencing analysis of each individual CpG sites for your chosen gene is conducted and provides a percent measure of methylation for each sample. The resulting methylation data can then be analyzed to look for trends and statistically significant findings. For our study, we chose to outsource the bisulfite modification, PCR amplification, and pyrosequencing to EpigenDX, a company specializing in this type of analysis based on Worcester, MA. For our statistical analysis and graphing, we used STATA™ Data analysis and statistical software.

After analyzing the patient samples in our initial pool of 22 women, we concluded that our small number of samples was not sufficient to reach statistical significance. To overcome this limitation, we chose to combine our methylation data with another sample

set of 102 women provided by Kathleen Arcaro, a collaborator in this study. Unlike the human mammary epithelial cells that were cultured from reduction mammoplasty tissue for the initial analysis, the epithelial cells isolated for the methylation study in the Arcaro lab were from human breast milk samples. The Arcaro lab completed their own bisulfite modification and PCR amplification, but also utilized EpigenDX for pyrosequencing their samples. STATA™ was again used for the combined data analysis.

In summary, we propose that changes in promoter methylation of three genes, CRBP1, SFRP1, and RASSF1, may correlate to risk factors such as age, parity status, and family history. Also, we plan to analyze each individual CpG site for our three genes to see if patterns of methylation emerge for individual sites. Finally, we will combine our methylation data with methylation data collected from epithelial cells isolated from breast milk samples to see if there are correlations between the two sample sets.

Materials and Methods

Acquisition of Donor Samples

Female patients undergoing reduction mammoplasty surgery at Baystate Medical Center in Springfield, Massachusetts were asked prior to their procedure to donate a portion of their removed breast tissue by their treating physician or physician assistant. After surgery, consenting patient tissue was collected within one hour of surgery and reviewed by a pathologist before being released to researchers at the Pioneer Valley Life Sciences Institute. Each patient was assigned a random number to protect their identity. Within 30 days of the procedure, patients were administered a questionnaire by an outside research personnel. The use of human subjects, which included informed

consent, was approved by the University of Massachusetts, IRB Human Research Protection Office (HRPO) and the Baystate Medical Center IRB office. A summary of the epidemiological data for the 22 patients included in this study is provided in Table 5.1.

Mammary Tissue Processing

Tissue was acquired by research personnel from the Pathology department at Baystate Medical Center and brought to the laboratory at the Pioneer Valley Life Sciences Institute in Springfield, Massachusetts. Tissue was finely minced and digested overnight in mammary digestion media. Any undigested tissue was removed and the digestion solution was centrifuged at 80 x g for 10 minutes to collect a mammary epithelial cell pellet. The pellet was then washed in 10 mL of HF epithelial wash and centrifuged. The pellet was then incubated with 2 mL of trypsin/EDTA for 5 minutes at room temperature and the HF wash and centrifugation were repeated. The pellet was then treated with 2 mL dispase and 10 μL DNase I for 5 minutes at room temperature and then HF wash and centrifugation was repeated. Cells were then passed through 100 μm and 40 μm cell strainers and centrifuged for 5 minutes at 100 x g. Cell pellet was then resuspended in 1 mL of mammosphere media, counted, and plated at a density of 20,000 cells per 10 cm ultralow attachment plate. Plates were labeled with patient #, date of harvest, and date of culture.

Cell Culture

Human mammary epithelial cells (HMECs) were cultured in MEGM supplemented with bovine pituitary extract for 12-14 days in a 37°C humidified incubator

with 5% CO2. Plates were examined daily, with the exception of most weekend days, for the growth of fibroblast cells that would contaminate the epithelial cell population. Upon examination, if fibroblast count reached approximately 30% of the total cell population, then the plate was discarded. Cells were grown to approximately 70% confluency prior to harvesting, but growth rates varied widely between patients and some patient cell cultures never reached this percentage of confluency and were instead harvested on day 14.

DNA Isolation

Human mammary epithelial cells were harvested either once they reached approximately 70% confluency or when day 14 of culture was reached, which ever came first. Cells were detached from their plates using trypsin, growth media was added to reduce stress on cells during harvesting, cells were centrifuged to form a pellet, trypsin and media was removed, and the pellet was resuspended in 200 μL of 1XPBS. All patient human mammary epithelial cell DNA was isolated using a Qiagen DNeasy Blood and Tissue Kit® and kit procedures were followed for isolation. After DNA was isolated, DNA quality and yield was measured via spectroscopy. Final DNA yield ranged widely from 30ng/μL to 524ng/μL. DNA samples were labeled with the patient's randomly assigned number and DNA concentration and were then overnighted to EpigenDX for analysis.

Bisulfite modification & Pyrosequencing

Bisulfite modification, PCR amplification, and pyrosequencing analysis were all conducted by EpigenDX in Worcester, MA. Methylation data was analyzed using regression analysis and graphed using STATA™ Data analysis and statistical software.

Results

Mean Methylation by CpG Site

Due to the small sample size, there are no statistically significant findings from the patient tissue samples, but there are interesting observations to be made. It has been demonstrated that certain CpG sites are more susceptible to methylation than others (Ref). Based on this, our first analysis was to examine the mean methylation scores for individual CpG sites for each gene. For CRBP1, although there are 19 total CpG sites, the pyrosequencing was unable to accurately collect methylation data past site 8, so we only examined the first 8 sites in our analysis. Examination of the individual CpG site percent methylation ranges revealed that overall CRBP1 methylation was low (below 5%) in our patients (Figure 5.1). Only three CpG sites had a patient whose score was over 5%, sites 3, 6, and 8. When you compare the patient profiles in terms of risk factors for the sample over 5% there is no pattern to them. What this suggests is that these three sites have variance with respect to CRBP1 methylation and could warrant further investigation, although the results of this study were inconclusive due to our small sample size.

SFRP1 had more methylation overall when compared with CRBP1. Every CpG site had at least one patient over 5% methylation and several sites had 3 or more patients

over 5% (sites 2 , 5 , 6, 7) (Figure 5.2). Similar to CRBP1, when you compare the individual patients between the CpG sites there is no pattern that emerges. Women that were highly methylated in one site were not in others and there were no risk factor groupings. Like SFRP1, RASSF1 had an overall higher methylation than CRBP1. Again, there are several sites of interest (sites 3, 5, 6 ,7, 8), but patient comparisons do not reveal interesting trends (Figure 5.3). Our low sample numbers limited the statistical analysis of our data and to fully explore if the sites with methylation above 5% for three or more patients are important the experiment would need to be repeated with additional patients.

Risk Factors and Mean Methylation Analysis

After examining mean methylation by CpG site for each gene, we turned to examine how risk factors may impact mean methylation for each gene. First, we investigated if current age had an effect on mean methylation. As is shown in Figure 5.4, there is no significant trend. We had hypothesized that as women age they would accumulate more methylation, but our data does not support that proposition. When you examine the methylation by gene, CRBP1 has low methylation overall and increasing age has no effect on mean methylation (Figure 5.5). SFRP1 also has low methylation overall, but the only two patients having methylation greater than 5% are over the age of 45 (Figure 5.6). Although this is an interesting observation, our low patient numbers do not make this finding significant, but do suggest that further investigation might be warranted. Finally, examination of RASSF1 mean methylation versus current age does not reveal any interesting trends (Figure 5.7). Increased patient numbers, and perhaps an

increase in patient over 50 years of age, should be considered if this analysis is to be repeated more accurately.

After examining the affect of age on methylation, we turned to the role of pregnancy generally and early pregnancy on mean methylation for each gene. We hypothesized that women who have had an early full-term pregnancy should have lower mean methylation scores in comparison with women who have not. It is important to note that the number of patients who had ever had a full-term pregnancy was only 8 out of the 22 total patients whom we have data on. First, we analyzed how the number of live births a woman has undergone impacted mean methylation scores for all three genes (Figure 5.8). Overall, methylation scores were below 5% and the only similarities between the women above 5% were that two of them had two pregnancies each and also have high methylation for SFRP1.

Pregnancy alone is not enough to garner protection against breast cancer. The age of the women during the time of her first pregnancy is important because studies have documented that women who undergo a full-term pregnancy before the age of 24 have a lifetime decreased risk of developing the (Russo et al. 2005). In order to account for this, we then analyzed the age of the women at the time of their first pregnancy versus the mean methylation score (Figure 5.9). Again, overall methylation scores were low, but the two patients that had high methylation scores in the first analysis were shown to be age 26 and 32 respectively at the time of their first pregnancy. Although these ages are not in the protective range, they are also not above age 35 and consequently at a higher risk. It is difficult given the limited sample size to draw conclusions about this observation regarding SFRP1 analysis, but it was important to point out.

Finally, family history of breast cancer is another important risk factor we chose to examine in this study. When comparing women who have no family history with women who have either a first or second degree relative with the disease in terms of their mean percent methylation scores, no interesting pattern emerges (Figure 5.10).

Combined Epithelial Cell Data Analysis

In an effort to supplement the limited mammoplasty tissue samples in our study, we combined our methylation data set of 22 patients with methylation data from 102 additional samples that were also pyrosequenced for the same three genes by EpigenDX. Our collaborator, Kathleen Arcaro, conducted pyrosequencing analysis on epithelial cells collected and purified from breast milk samples from lactating women. Similar to our study, we received current age and parity status information on these additional samples.

After combining the two data sets, we wanted to investigate whether the source of the mammary epithelial cells had any effect on mean methylation scores. First a regression analysis was completed for RASSF1 for cell source while controlling for current age with 124 total samples. What this analysis showed was that cells derived from the milk samples had a significantly higher mean methylation score than cells derived from the reduction mammoplasty tissue (P value 0.033). Current age did not exhibit a significant trend. As is shown in Figure (5.11), graphing the mean methylation scores for RASSF1 over the cell source reveals a number of very high methylation scores that could have been confounding our results. In order to demonstrate that the higher mean methylation scores for cells derived from the milk samples were accurate, the regression was performed again removing all methylation scores above 10.31 (or 8

samples). Removing the samples above 10.31% methylation only strengthened our finding that epithelial cells derived from milk samples have a higher mean methylation score than cells derived from the reduction mammoplasty tissue (P value 0.029). Finally, although current age is an important variable to control for in the regression analysis, we hypothesized that parity may also play an important role in mean methylation scores in that based on the role of parity-induced protection, parous women might have lower mean methylation scores that nulliparous women. Given this possible confounding of our significant findings, the regression analysis was repeated taking parity into account (119 samples). After controlling for both current age and parity status, epithelial cells derived from milk were still shown to have an even more significantly increased (P value 0.007) mean methylation scores when compared to cells derived from reduction tissue. Additionally, after controlling for parity status, there is an observed trend that current age may be impacting mean methylation (P value 0.075). Based on our results, parity status appears to play no significant role in mean methylation score.

After observing that epithelial cells derived from breast milk samples had higher mean methylation scores than those from reduction mammoplasty tissue, the analysis was repeated for CRBP1. First a regression analysis was completed for cell source while controlling for current age with 116 samples. Similar to RASSF1, cells derived from the milk samples had a significantly higher mean methylation score (P value 0.00). Current age did not exhibit a significant trend. As is shown in Figure 5.12, graphing the mean methylation scores for CRBP1 over the cell source reveals a number of very high methylation scores that could have been confounding our results. In order to demonstrate that the higher mean methylation scores for cells derived from the milk samples were

accurate, the regression was performed again removing all methylation scores above 10.15 (or 5 samples). Epithelial cells derived from milk samples once again had a significant increase in mean methylation scores (P value 0.00) versus cells derived from the reduction mammoplasty tissue. Finally, although current age is an important variable to control for in the regression analysis, we hypothesized that parity may also play an important role in mean methylation scores in that parous women should have lower mean methylation scores that nulliparous women. Given this possible confounding of our significant findings, the regression analysis was repeated taking parity into account (113 samples). After controlling for both current age and parity status, epithelial cells derived from milk were still shown to have significantly increased (P value 0.00) mean methylation scores when compared to cells derived from reduction tissue. For CRBP1, there was no significant trend for current age or parity having an effect of mean methylation scores.

After observing that epithelial cells derived from breast milk samples had higher mean methylation scores than those from reduction mammoplasty tissue for both RASSF1 and CRBP1, the analysis was repeated for SFRP1. Initially, a regression analysis was completed for cell source while controlling for current age with 122 samples. Similar to RASSF1 and CRBP1, cells derived from the milk samples had a higher mean methylation score that was significant (P value 0.048). Current age did not exhibit a significant trend. As is shown in Figure 5.13, graphing the mean methylation scores for SFRP1 over the cell source reveals a number of high methylation scores that could have be outliers confounding our results. In order to demonstrate that the higher mean methylation scores for cells derived from the milk samples were accurate, the

regression was performed again removing all methylation scores above 13.76 (or 7 samples). Epithelial cells derived from milk samples once again had a significant increase in mean methylation scores (P value 0.011) versus cells derived from the reduction mammoplasty tissue. As was explained earlier, although current age is an important variable to control for in the regression analysis, parity is another risk factor for breast cancer that could account for the higher mean methylation scores we observed. Given this possible confounding of our significant findings, the regression analysis was repeated taking parity into account (118 samples). After controlling for both current age and parity status, the trend of epithelial cells derived from milk having a significantly increased mean methylation scores when compared to cells derived from reduction tissue was lost (P value 4.60). For SFRP1, there was no significant trend for current age or parity having an effect of mean methylation scores. In conclusion, parity status, although it does not significantly impact mean methylation scores in it of itself, controlling for parity does result in a loss of difference in mean methylation scores we observed between epithelial cells derived from milk and those derived from reduction mammoplasty tissue.

Discussion

In this experiment, we proposed that the amount of CpG promoter methylation of RASSF1, CRBP1, and SFRP1 would vary with respect to specific CpG sites and that promoter methylation is related to breast cancer risk factors such as parity status, age, and family history of the disease. Although our data did indicate differing levels of mean percent methylation for individual CpG sites for each of the genes we examined, our sample numbers were too low to make strong conclusions about these observations. In terms of the risk factors we examined, although there were some interesting findings,

overall the methylation scores for the patients we analyzed were low (below 5%) and no significant trends could be indentified relating specific risk factors to increased methylation. Although significance was not reached in this experiment, this study did introduce a new technique for methylation analysis to the lab and paves the way for future patient studies that can build upon our currently limited data. Also, we were able to observe several challenges inherent to relying upon patient data and culturing HMEC cells that will be helpful to other researchers in our lab.

There were several major challenges we experienced when completing this experiment. The study relies upon women undergoing breast reduction surgeries at Baystate Hospital in Springfield, MA consenting to donate their tissue and being willing to complete a survey to collect personal information afterwards. As is evident upon examining Table 5.1, there were many women who initially consented to the study, so their tissue was collected and their cells were put into culture, but later failed to complete the survey. This resulted in several patients being included in the study with only age-related information, the one piece of data we have on all patients at the time of tissue harvesting. Although this did not inhibit our analysis of age in the study, it did drastically reduce the number of patients in our analysis of parity status and family history in promoter methylation. Given our relatively small sample number, these reductions in usable patient data negatively impacted our statistical analysis. To account for this problem in future experiments, the number of patients needed should be overestimated to account for failure of some patients to complete the needed personal information survey.

Another challenge we experienced in conducting this experiment was working with the human mammary epithelial cells (HMECs) isolated from the reduction

mammoplasty tissue. HMEC cells were difficult to culture. Several of our initial patients we had hoped to include in the study had to be excluded because their cells died in culture or their mammary epithelial cells became overrun with fibroblasts while in culture. The tissue digestion process used to isolate the mammary epithelial cells did not have a mechanism for enriching for this particular cell population, resulting in a mix of epithelial cells and fibroblasts being plated. In addition to being difficult to maintain in culture, variance in growth rates, and the mixed cell population, DNA yields and purity varied widely and at times were not sufficient for the necessary bisulfite modification and pyrosequencing requirements for the study. Initially, we had intended to complete the bisulfite modification to covert all unmethylated cytosines to uracil, a necessary step prior to pyrosequencing, in the lab. Although we successfully bisulfite modified several patient samples, the process resulted in the loss of overall DNA yield. Given the relatively low amount of DNA isolated from the cells and the fact that for many patients we only had one plate that was available to harvest and could not pool patient DNA from multiple plates this posed a major problem and caused us to outsource our bisulfite modifications and PCR amplifications to EpigenDX, who was able to complete the modifications with smaller amounts of DNA.

One of the most interesting observations to come out of this study is that human mammary epithelial cells derived from reduction mammoplasty tissue and those derived from breast milk were significantly different with respect to mean percent methylation. There are three major differences between the mammoplasty epithelial cells and the breast milk epithelial cells that could explain this finding. The first difference is the cellular environment at the time of collection and method of cell isolation. The

mammoplasty epithelial cells were isolated and cultured from intact tissue for up to two weeks and there was no enrichment for the epithelial cell population. The breast milk cells were exfoliated by the breast during lactation and were collected and purified directly from the milk samples and were never grown in culture. Also, they were able to enrich for the epithelial cells through epithelial-specific MACS HEA-125 microbeads (Miltenyi Biotec, Germany). Another difference to consider between the two cell populations is that all of the women in the milk sample have had at least one full-term pregnancy and were lactating at the time the milk samples were collected in contrast with the more varied population in reduction mammoplasty pool. Finally, the Arcaro lab completed their own bisulfite modification and PCR amplification of the DNA prior to sending the samples to EpigenDX while our samples were sent as unmodified DNA to EpigenDX and they completed the bisulfite modification, PCR amplification, and pyrosequencing. It is possible that any of these differences between the two sample sets could explain our observation that milk cells are significantly different than mammoplasty cells with respect to mean methylation.

In conclusion, although there were limited statistically significant findings in this study, a new technique was successfully introduced to our lab and interesting observations were made about CpG promoter methylation of CRBP1, SFRP1, and RASSF1 that can be built upon by other researchers. Additional investigation into the specific CpG sites targeted by hypermethylation in each of these three genes is needed and the relationship between breast cancer risk factors and methylation also requires further examination. Our hypotheses in this study were not confirmed, but instead, remain inconclusive.

Table 5.1 Summary of patient epidemiological data

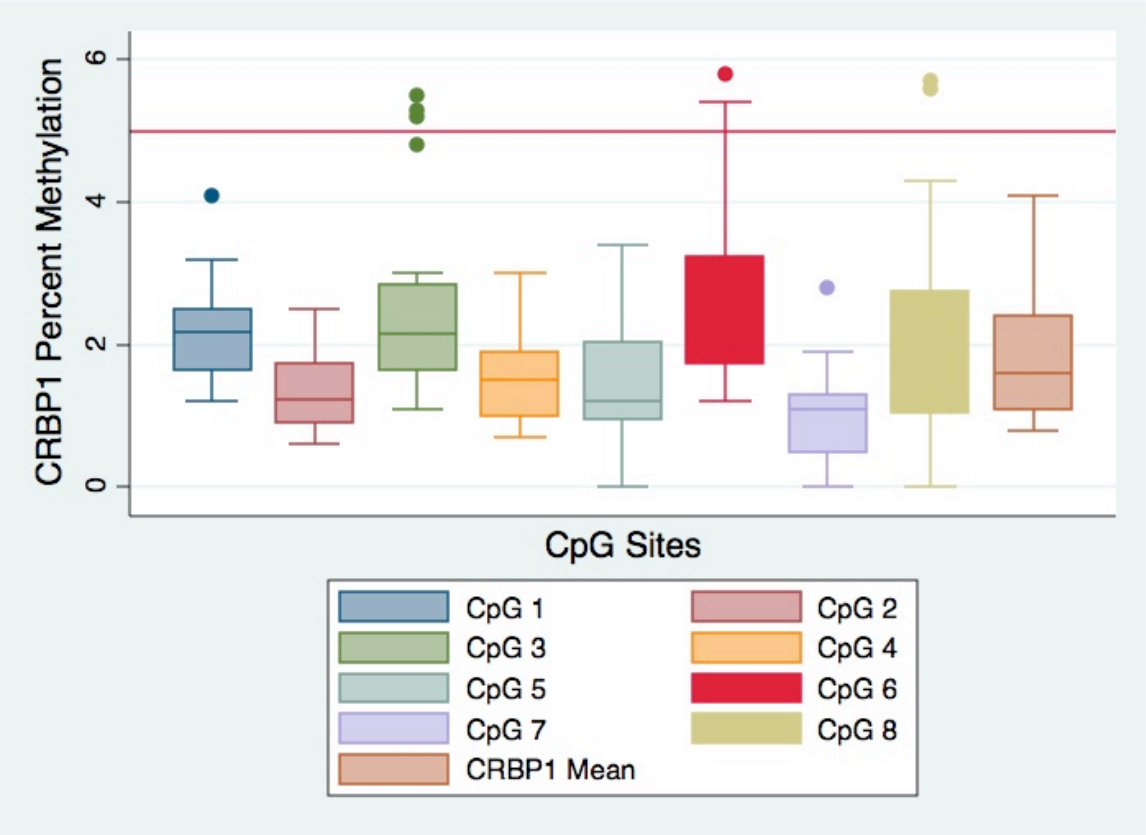
Summary of Patient Data					
#	**Current Age**	**GRAVA**	**PARA**	**Age at 1st Birth**	**Family History**
1	54				
2	17	0	0	0	No
3	29	0	0	0	No
4	21	0	0	0	Yes (2°)
5	38				
6	27	2	0	0	Yes (2°)
7	16	0	0	0	Yes (2°)
8	34	0	0	0	No
9	23	1	1	17	No
10	52	3	3	18	No
11	55	2	2	32	Yes (1°)
12	44	4	3	30	Yes (2°)
13	56				
14	35				
15	56				
16	18	0	0	0	No
17	34	1	1	30	Yes (1°)
18	50	0	0	0	No
19	39	1	1	27	No
20	59	4	3	19	No
21	25	1	0	0	No
22	49	3	2	26	No

Blank cells indicate that data is missing

1°= First degree relative with breast cancer (parent, sibling, offspring)

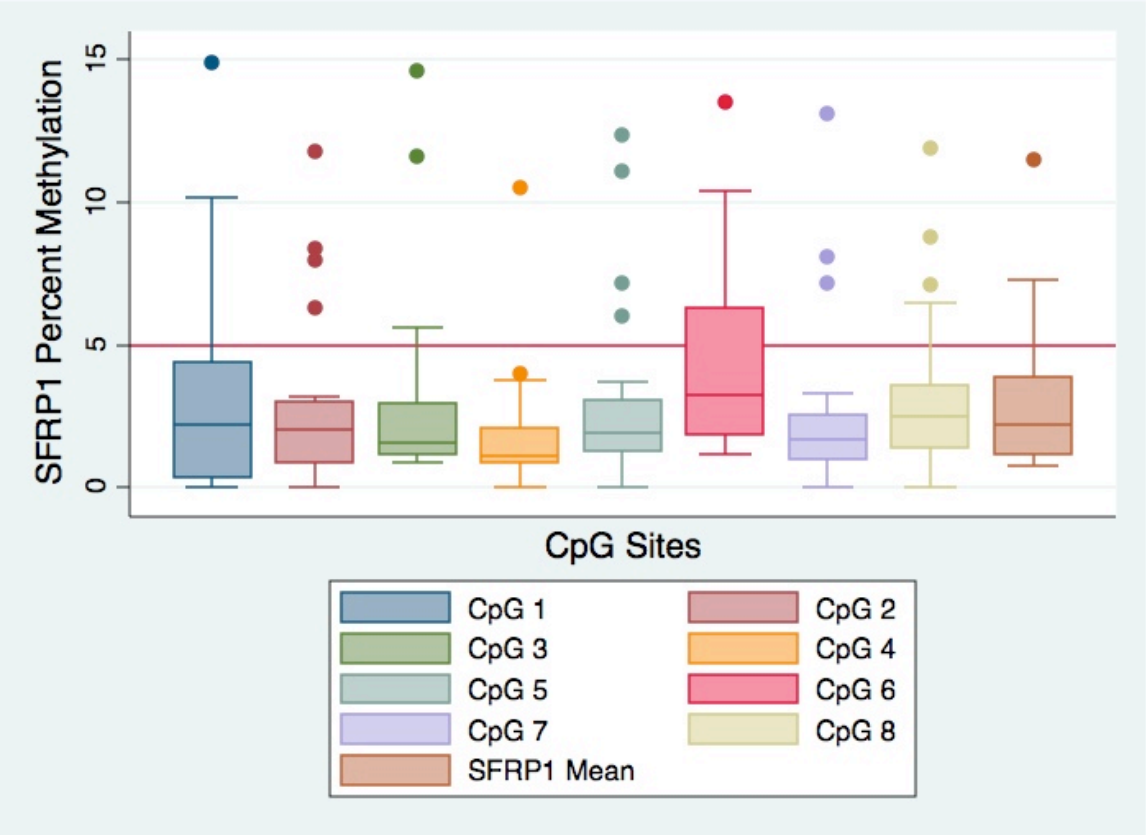
2°= Second degree relative with breast cancer (grandparent, aunt, niece, half-sibling)

Figure 5.1 CpG site-specific analysis for CRBP1 promoter methylation



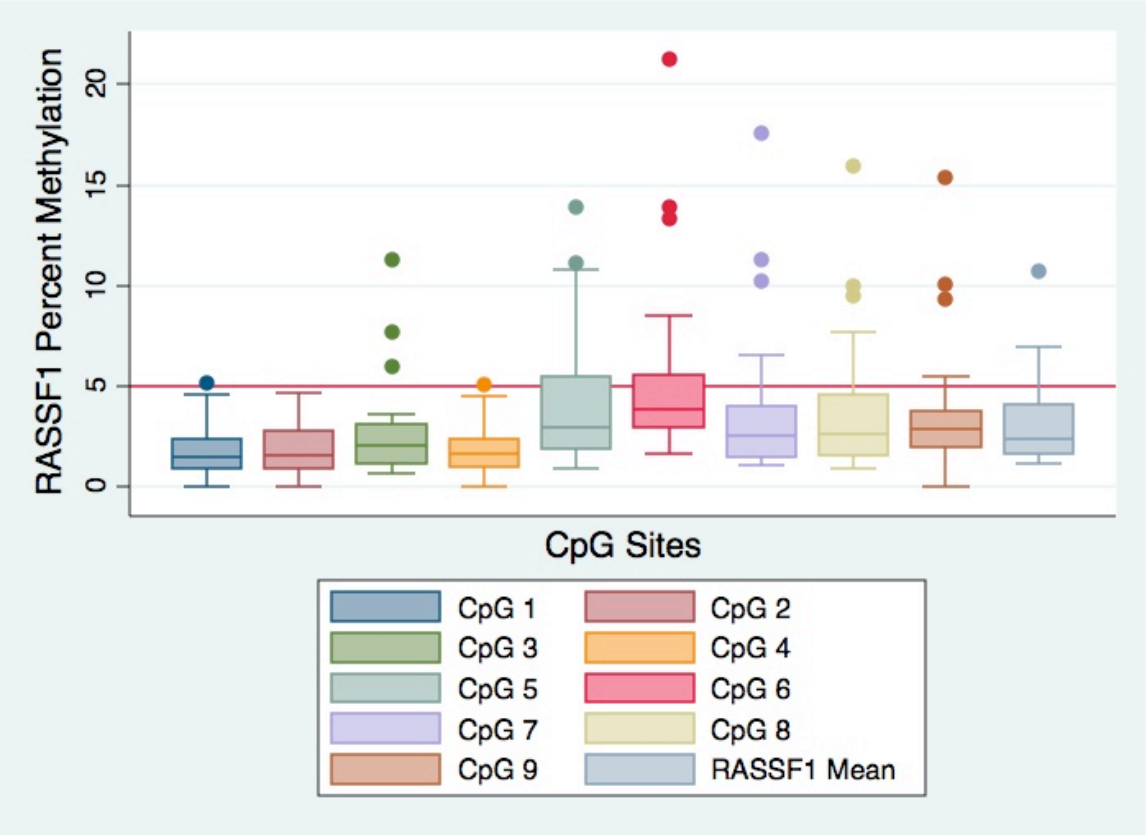
Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.2 CpG site-specific analysis for SFRP1 promoter methylation



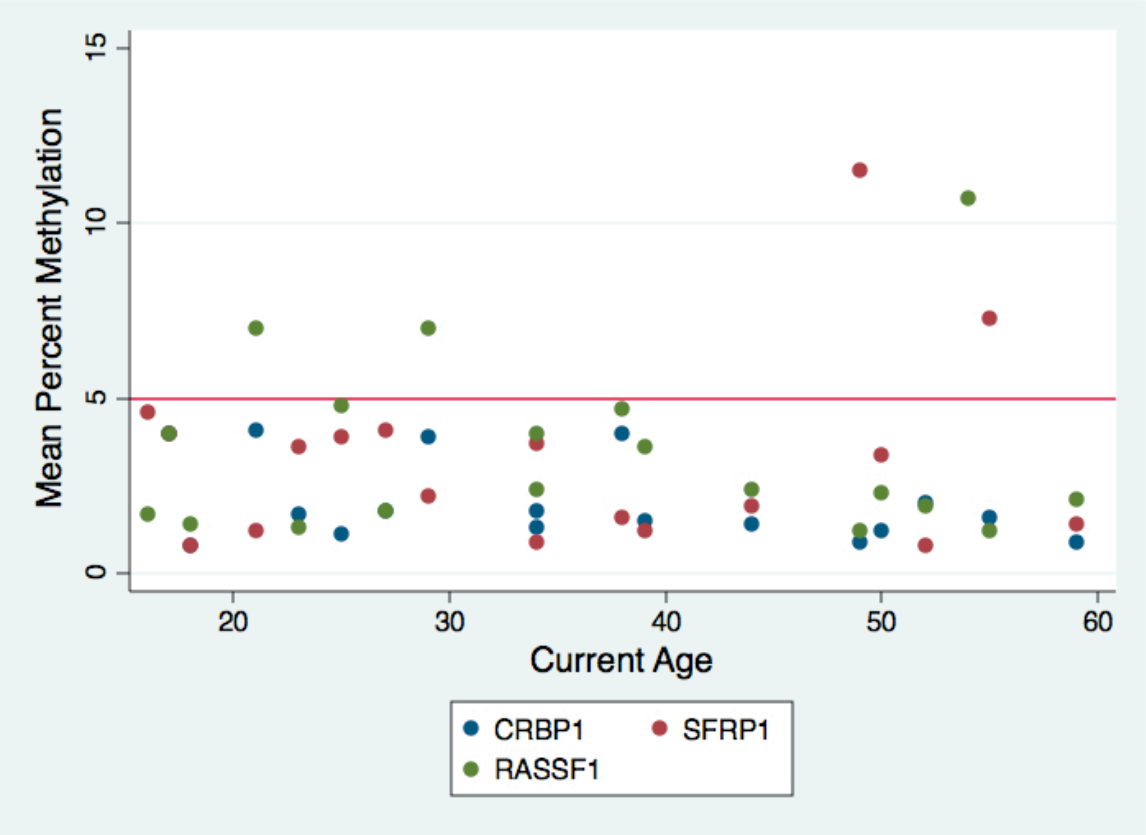
Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.3 CpG site-specific analysis for RASSF1 promoter methylation



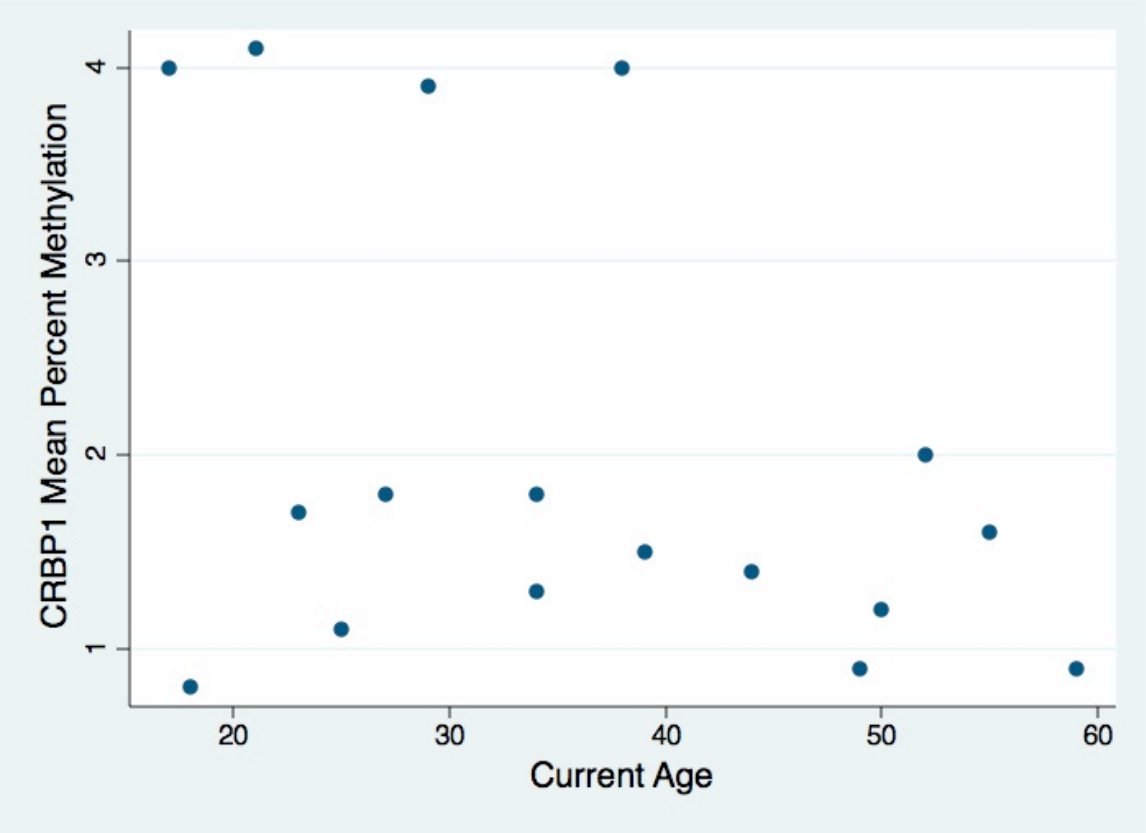
Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.4 Examination of how current age may affect CpG mean percent methylation of CRBP1, SFRP1, and RASSF1



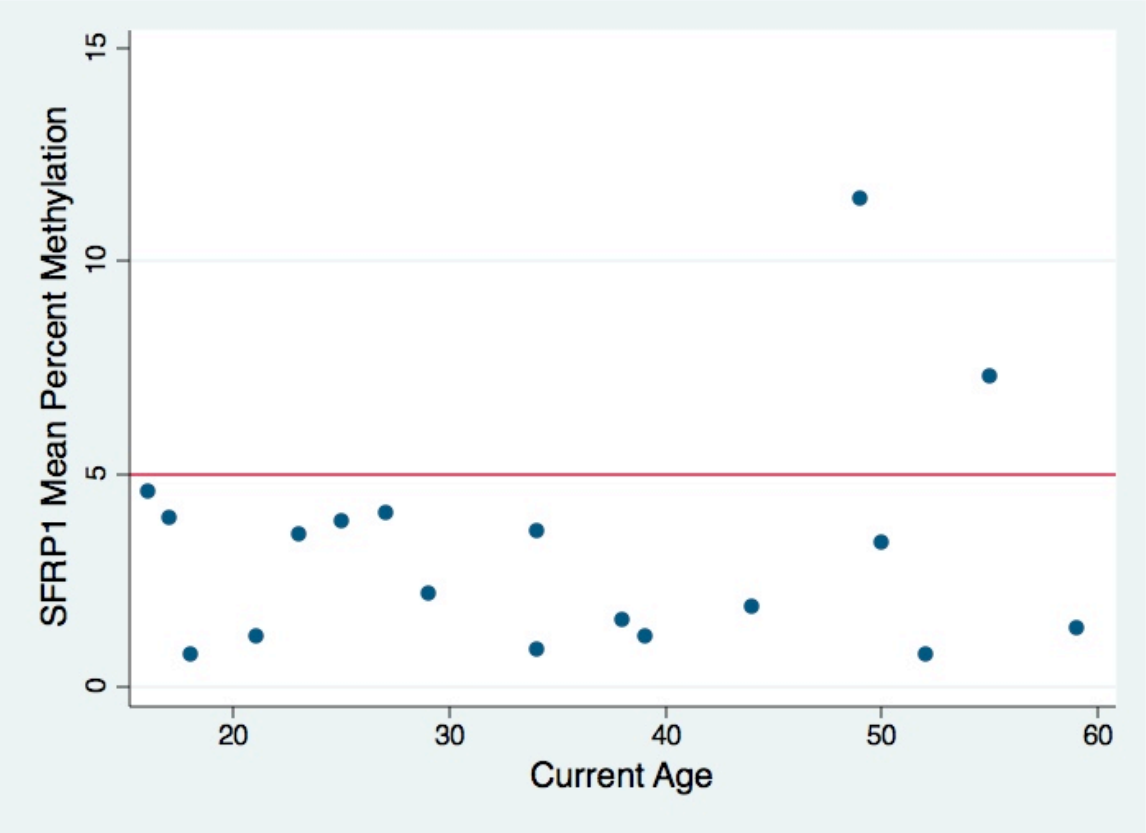
Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.5 Examination of how current age may affect CpG mean percent methylation of CRBP1



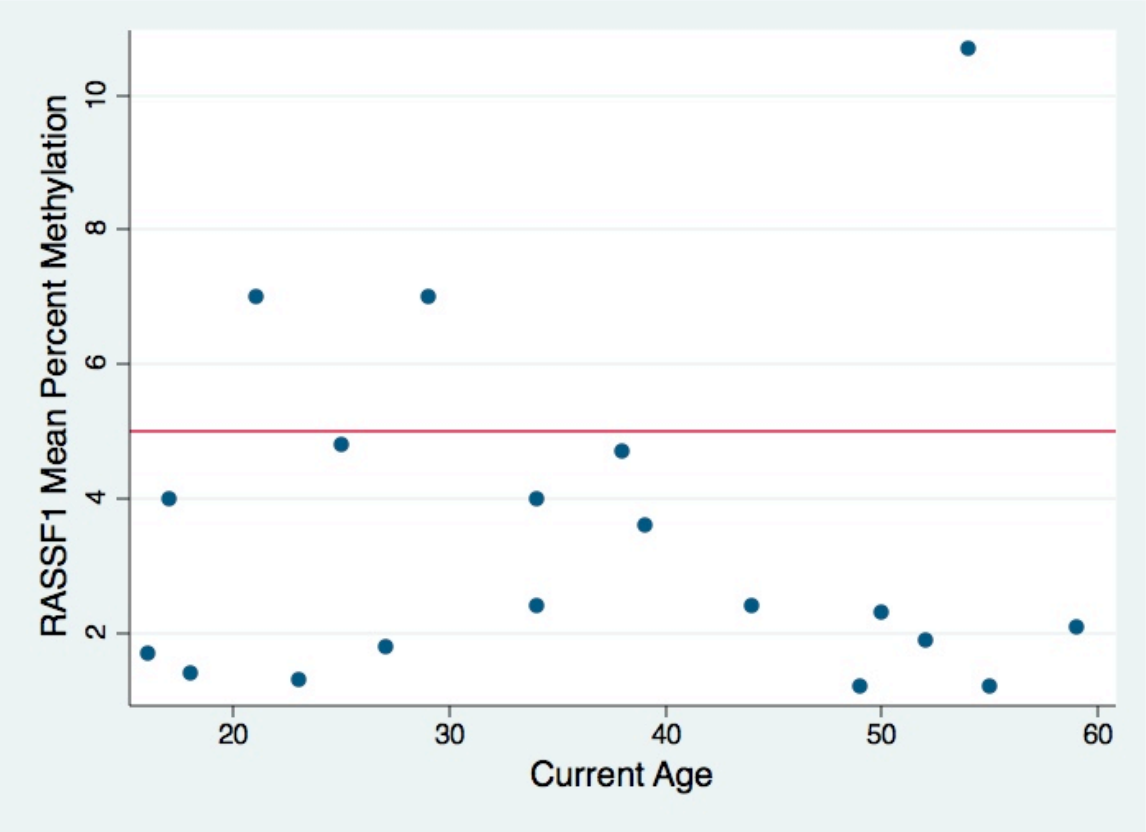
Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.6 Examination of how current age may affect CpG mean percent methylation of SFRP1



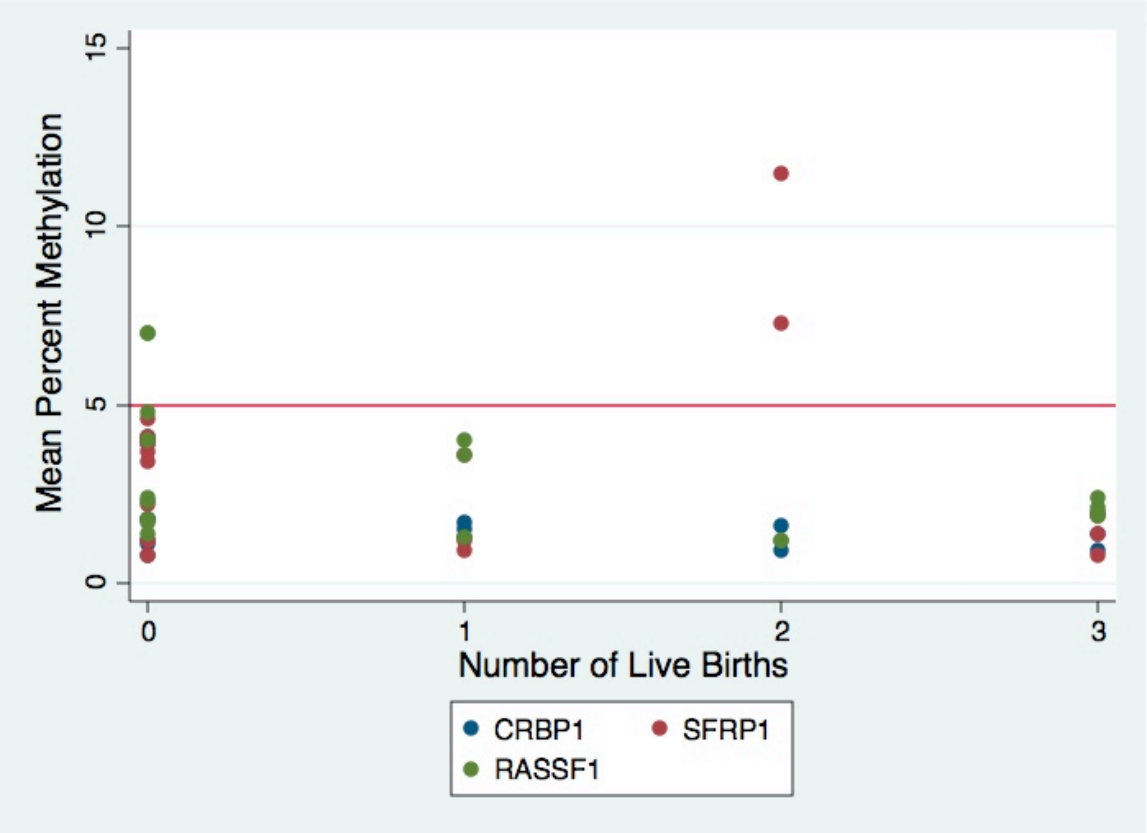
Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.7 Examination of how current age may affect CpG mean percent methylation of RASSF1



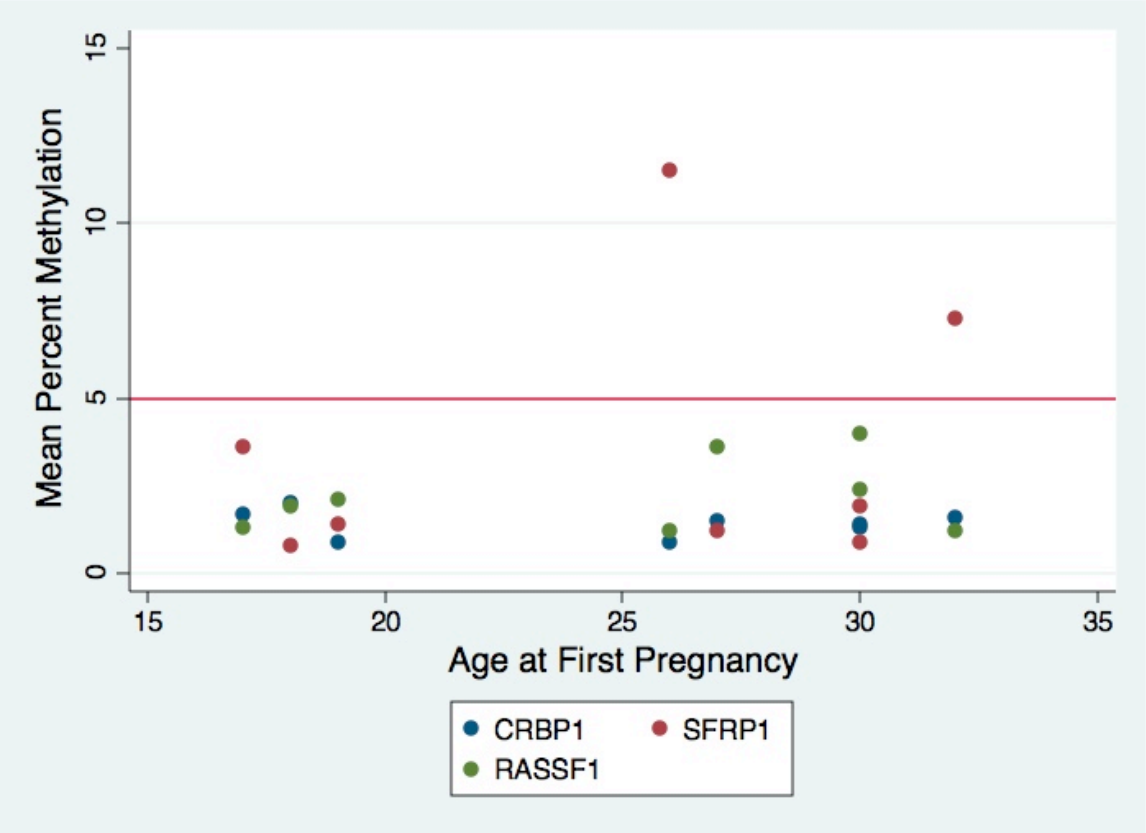
Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.8 Examination of how number of live births may affect CpG mean percent methylation of CRBP1, SFRP1, and RASSF1



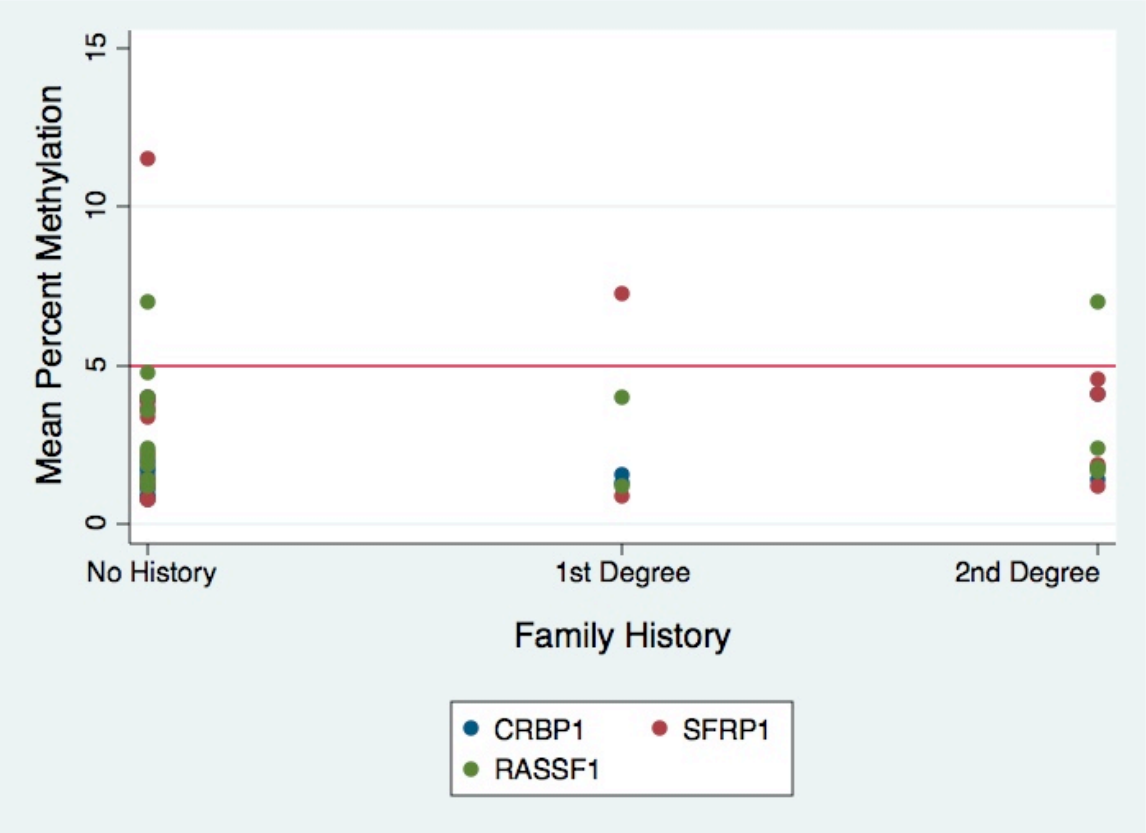
Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.9 Examination of how age at first pregnancy may affect CpG mean percent methylation of CRBP1, SFRP1, and RASSF1



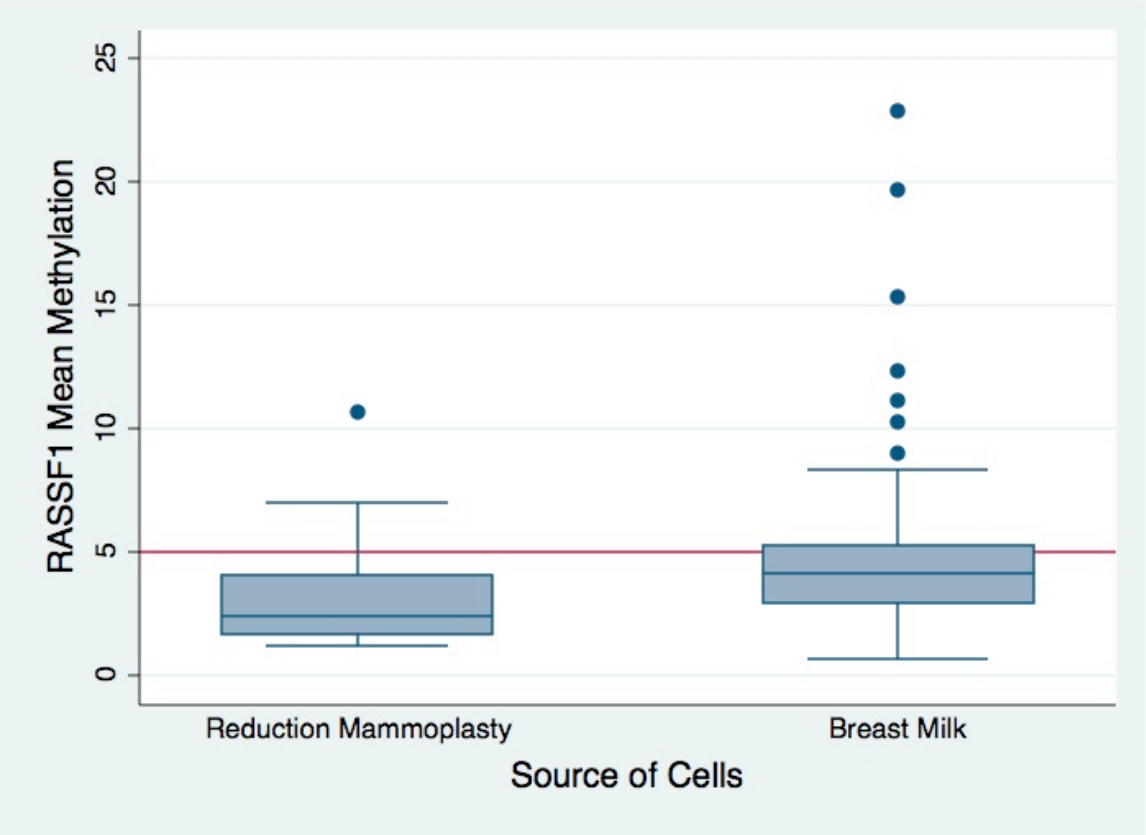
Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.10 Examination of how family history may affect CpG mean percent methylation of CRBP1, SFRP1, and RASSF1



Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.11 Examination of how source of mammary epithelial cells from either reduction mammoplasty tissue or breast milk affects RASSF1 mean methylation

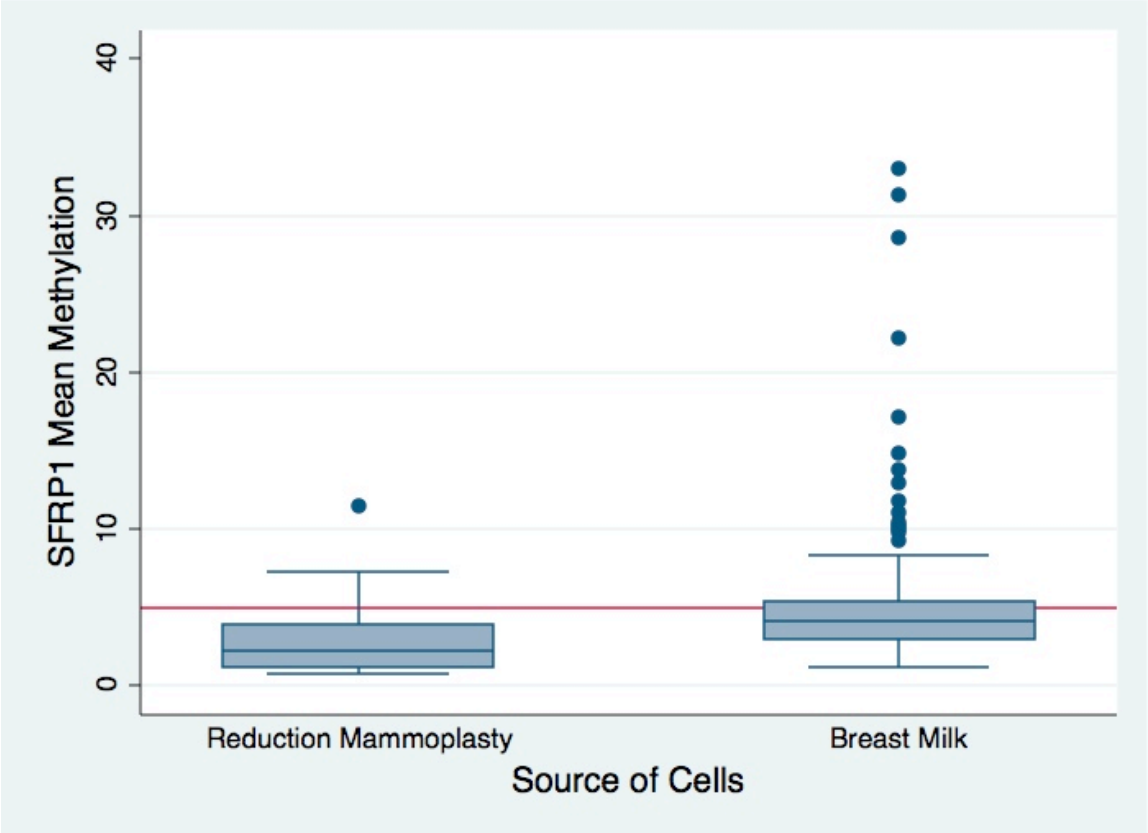


Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.12 Examination of how source of mammary epithelial cells from either reduction mammoplasty tissue or breast milk affects CRBP1 mean methylation

Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

Figure 5.13 Examination of how source of mammary epithelial cells from either reduction mammoplasty tissue or breast milk affects SFRP1 mean methylation



Red bar indicates 5% methylation. Samples below the line are considered to have low levels of methylation.

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